Virology 410 (2011) 64–75

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Epstein–Barr Virus nuclear antigen 1 (EBNA1) confers resistance to apoptosis in EBV-positive B-lymphoma cells through up-regulation of survivin

Jie Lu [a], Masanao Murakami [a], Subhash C. Verma [a], Qiliang Cai [a], Sabyasachi Haldar [a], Rajeev Kaul [a], Mariusz A. Wasik [b], Jaap Middeldorp [c], Erle S. Robertson [a,*]

[a] Department of Microbiology and the Tumor Virology Program, Abramson Comprehensive Cancer Center, School of Medicine, University of Pennsylvania, 202E Johnson Pavilion, 3610 Hamilton Walk, Philadelphia, PA 19104, USA

[b] Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

[c] Department of Pathology, Cancer Center Amsterdam, Vrije Universiteit University Medical Center, 1081HV Amsterdam, The Netherlands

ARTICLE INFO

Article history:
Received 8 July 2010
Returned to author for revision 10 August 2010
Accepted 18 October 2010
Available online 19 November 2010

Keywords:
EBNA1
Survivin
Sp1
Apoptosis
Oncogenic process

ABSTRACT

Resistance to apoptosis is an important component of the overall mechanism which drives the tumorigenic process. EBV is a ubiquitous human gamma-herpesvirus which preferentially establishes latent infection in viral infected B-lymphocytes. EBNA1 is typically expressed in most forms of EBV-positive malignancies and is important for replication of the latent episome in concert with replication of the host cells. Here, we investigate the effects of EBNA1 on survivin up-regulation in EBV-infected human B-lymphoma cells. We present evidence which demonstrates that EBNA1 forms a complex with Sp1 or Sp1-like proteins bound to their cis-element at the survivin promoter. This enhances the activity of the complex and up-regulates survivin. Knockdown of survivin and EBNA1 showed enhanced apoptosis in infected cells and thus supports a role for EBNA1 in suppressing apoptosis in EBV-infected cells. Here, we suggest that EBV encoded EBNA1 can contribute to the oncogenic process by up-regulating the apoptosis suppressor protein, survivin in EBV-associated B-lymphoma cells.

Introduction

EBV is a human gamma-herpesvirus known to establish latent infections in multiple cell types and is associated with a large number of malignancies, including Burkitt's lymphoma (BL), nasopharyngeal carcinoma (NPC), Hodgkin's disease (HD), and posttransplant lymphoproliferative disease (PTLD) (Young and Rickinson, 2004). Some EBV-positive tumors express only a limited number of latency proteins, leading to restricted forms of latency in which Epstein–Barr Virus nuclear antigen 1 (EBNA1) is detected either alone (Latency I, found in BL) or together with the LMPs (Latency II, found in HD and NPC, and Latency III, found in PTLDs and LCLs) (Leight and Sugden, 2000; Rickinson and Kieff, 2001). Thus, EBNA1 is detected in all types of malignancies positive for EBV. Moreover, EBNA1 is required for maintenance of the EBV episome (Hung et al., 2001). This finding is consistent with a central role for EBNA1 in maintenance and segregation of the latent EBV genome (Imai et al., 1998; Rickinson and Kieff, 1996). EBNA1 also promotes genomic instability via induction of reactive oxygen species (Gruhne et al., 2009) and in complex with EBNA1-binding protein 2 (EBP2) can interact with mitotic chromosomes (Nayyar et al., 2009).

As a DNA-binding phosphoprotein, EBNA1 binds to the origin of viral replication, oriP, critical for latent viral replication and for tethering of the viral episome to host chromosomes (Marechal et al., 1999; Yates et al., 1996). Recently, Frappier L. et al. reported that EBNA1 mediated recruitment of a histone H2B deubiquitylating complex to OriP, the latent origin of DNA replication (Sarkari et al., 2009). EBNA1 also interacts with the Bromodomain containing Protein 4 (Brd4) in both yeast and human cells through N-terminal sequences. This induces transcriptional activation but not segregation (Lin et al., 2008). Furthermore, EBNA1 participates in regulation of latent gene transcription as EBNA1 can bind to a site downstream of the BamHI Q promoter (Qp) to repress Qp activity (Sung et al., 1994). EBNA1 has also been shown to up-regulate two key EBV latency promoters, the BamHI C promoter (Cp) (Sugden and Warren, 1989) and the LMP1 promoter (Gahn and Sugden, 1995). Furthermore, EBNA1 enhances the activity of the AP-1 transcription factor in NPC (O'Neil et al., 2008), and is capable of transactivating the Wp/Cp promoters responsible for initiation of transcription of the six EBNAs in type III latency (Imai et al., 1998; Rickinson and Kieff, 1996). EBNA1 can also regulate cellular gene expression (Canaan et al., 2009) and inhibits the canonical NF-κB pathway by inhibiting IKK phosphorylation, implicating EBNA1 in the pathogenesis of NPC (Valentine et al., 2010). Moreover, expression of EBNA1 in gastric carcinomas cells is associated with enhanced tumorigenicity (Cheng et al., 2010). In addition, a number of published studies are consistent with a role for EBNA1 in proliferation of

* Corresponding author. Fax: +1 215 898 9557.
E-mail address: erle@mail.med.upenn.edu (E.S. Robertson).

0042-6822/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2010.10.029

EBV-positive cells. For example, interference with EBNA1 function in EBV-positive Burkitt's lymphoma cells, by overexpression of a dominant-negative EBNA1 mutant resulted in increased cell death (Kennedy et al., 2003). Similarly, down-regulation of EBNA1 in Raji, a Burkitt's lymphoma cell line or EBV-positive epithelial cells by RNA interference decreased cell proliferation (Hong et al., 2006; Yin and Flemington, 2006). Our previous work have also shown that expression of EBNA1 in breast carcinoma cells promoted the rate of tumor growth in nude mice, reversed the growth inhibitory effect of the cellular Nm23-H1 protein and increased lung metastases (Kaul et al., 2007).

Survivin is an inhibitor of apoptosis expressed in many human cancer cells but not in normal adult tissues (Altieri, 2008; Hong et al., 2006). Overexpression of survivin in various cellular systems was associated with inhibition of cell death (Altieri, 2008). However, regulation of the survivin gene is complex, involving multiple pathways regulated by transcriptional and post-transcriptional strategies (Altieri, 2008). Survivin transcription increases during G_1, and reaches a peak in G_2-M (Kobayashi et al., 1999; Li and Altieri, 1999). Survivin has generated considerable interest as a cancer gene, although no mutations or polymorphisms have been identified that selectively induce survivin. Notably p53, or expression of oncogenes like Ras, results in aberrant survivin promoter activity (Altieri, 2003). Furthermore, the feasibility of utilizing the survivin promoter to obtain cancer-specific expression of therapeutic genes was exploited in a number of studies (Chen et al., 2004; Chun et al., 2007; Yang et al., 2004). In addition, survivin is regulated by the developmental signaling pathway as a direct transcriptional target of Wnt/β-catenin, which recognizes the discrete T-cell factor (TCF-4)-binding elements in the survivin promoter (Fodde and Brabletz, 2007). Recent studies have also shown that survivin is a direct transcriptional target of Notch-dependent gene expression which involves regulation of the RPB-Jκ-binding sites in the survivin promoter (Lee et al., 2008). Additionally, the transcription factor GATA-1 is overexpressed in breast carcinomas and contributes to survivin up-regulation via promoter polymorphism (Boidot et al., 2010). Recently up-regulation of survivin expression was due to expression of latent membrane protein 2A (LMP2A) in EBV-associated gastric carcinoma (Hino et al., 2008). However, the mechanism of this activation is yet to be fully elucidated.

Here, we investigated the effects of EBNA1 on survivin expression in EBV-infected B-lymphoma cells. We present evidence to suggest that EBNA1 forms a complex with Sp1 or Sp1-like proteins bound to their cis-element on the survivin promoter. This enhances the activity of these complexes and up-regulates survivin expression. Survivin up-regulation was therefore due to EBNA1 expression; and an increase in apoptosis due to knockdown of EBNA1 or survivin suggests that EBNA1 plays an important role in cell survival and proliferation. These results demonstrate that EBNA1 can contribute to the oncogenic process through inhibition of apoptosis in EBV-positive B-lymphoma cells.

Results

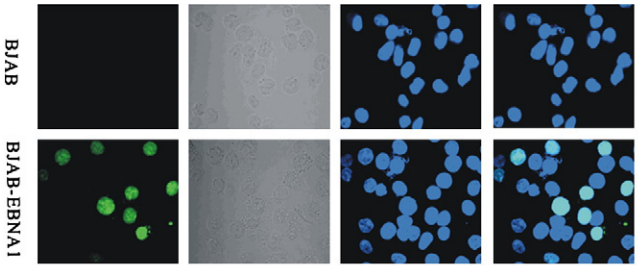
Survivin is up-regulated in BJAB cells expressing EBNA1

EBNA1 contributes to the replication of the EBV genome and is essential for persistence of the viral genome in host cells (Kapoor et al., 2005; Wu et al., 2000). In addition, EBNA1 has been reported to regulate cellular pathways through its activation as a transcription factor (Wood et al., 2007). In order to further determine the effects of EBNA1 on genes associated with a number of these cellular processes, we utilized a cell cycle specific array to determine the difference in mRNA levels with EBNA1 expression when compared to the control vector alone. Cells expressing EBNA1 showed modulation in the signal intensities of a large number of cellular genes. Similarly, spots of artificial biotinylated sequence used as controls showed similar levels of hybridization and confirmed that the biotin labeling was equally efficient in both BJAB-EBNA1 and BJAB-vector cDNA (data not shown). Gene array data acquisition and quantification of spot intensities in the membrane were performed by using GEArray Expression Analysis Suite 2.0. Only genes with changes greater than 2.2-fold were considered to be differentially expressed. These included BIRC5 (baculoviral IAP repeat-containing 5, or survivin), CCND3 (Cyclin D3), CDC20 (Cell division cycle 20 homolog, S. cerevisiae), CDKN1A (Cyclin-dependent kinase inhibitor 1A), CKS2 (CDC28 protein kinase regulatory subunit 2), CUL1 (Cullin 1), E2F1 (E2F transcription factor 1), KPNA2 (Karyopherin alpha 2), MAD2L2 (MAD2 mitotic arrest deficient-like 2), MCM2 (MCM2 minichromosome maintenance deficient 2, mitotin, S. cerevisiae), and UBE1 (Ubiquitin-activating enzyme E1) (Fig. 1B). The baculoviral IAP repeat-containing 5 (BIRC5), or survivin, was up-regulated about 3.3-fold above the cut-off (Fig. 1B). Interestingly, survivin belongs to the inhibitor of apoptosis protein (IAP) family and functions to inhibit caspase activity and therefore negatively regulates apoptosis (Tamm et al., 1998). We therefore chose to investigate the levels of survivin expressed in EBV-infected cells compared to control cells and explored the potential link between EBNA1 and survivin in EBV-associated B-lymphoma Cells.

The transcription and protein levels of survivin are induced by EBNA1

The mRNA and protein levels were analyzed and the results further supported the gene array data showing that survivin expression was up-regulated. We performed RT-PCR and Western blot analyses to show the effect of EBNA1 on the levels of mRNA and

A



B

Symbol	*Description*	*Fold change*	*RefSeq Number*
BIRC5	Baculoviral IAP repeat-containing 5 (survivin)	3.33	NM_001168
CCND3	Cyclin D3	2.90	NM_001760
CDC20	Cell division cycle 20 homolog (S. cerevisiae)	2.46	NM_001255
CDK4	Cyclin-dependent kinase 4	2.20	NM_000075
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	8.30	NM_000389
CKS2	CDC28 protein kinase regulatory subunit 2	2.72	NM_001827
CUL1	Cullin 1	3.22	NM_003592
E2F1	E2F transcription factor 1	7.46	NM_005225
KPNA2	Karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	2.59	NM_002266
MAD2L2	MAD2 mitotic arrest deficient-like 2 (yeast)	3.25	NM_006341
MCM2	MCM2 minichromosome maintenance deficient 2, mitotin (S. cerevisiae)	3.19	NM_004526
UBE1	Ubiquitin-activating enzyme E1	3.16	NM_003334

Fig. 1. Genes up-regulated in BJAB cells expressing EBNA1 by using gene array analysis. (A) Immunofluorescence assay for EBNA1 in BJAB cells. (B) The genes shown were up-regulated greater than 2.2-fold in BJAB cells expressing EBNA1. Data was standardized from BJAB-EBNA1 and BJAB-vector sets by using GEArray Expression Analysis Suite.

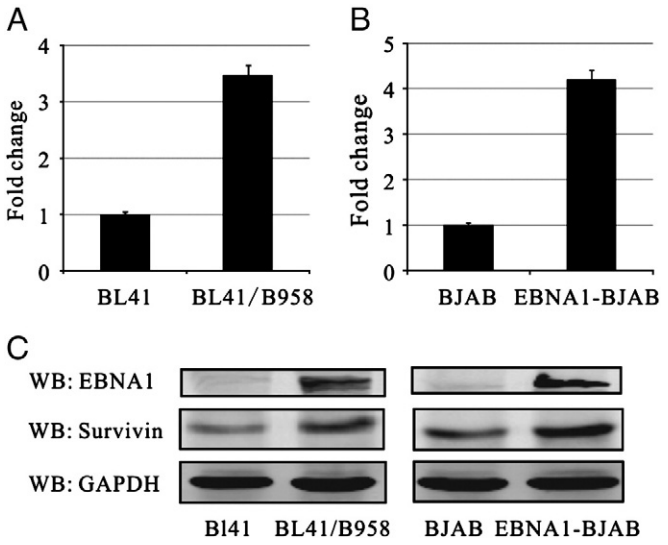


Fig. 2. The transcription and protein levels of survivin were induced by EBNA1. (A, B) Quantitation of survivin transcripts was measured by semi-quantitative RT-PCR. Total RNA was isolated from 20 million of EBV non-infected BL41 and infected BL41/B958 cells or BJAB, EBNA1-expressing BJAB cell by using TRIzol reagent. A total of 2 μg of RNA was used to synthesize cDNA. Real-time PCR was performed by using the Power SYBR green PCR master mix with GAPDH as control. Error bars show standard deviations; (C) Western blot analysis of endogenous survivin in the EBV-infected BL41 and BJAB cells expressing EBNA1. GAPDH was used for internal control.

protein. We extracted total RNA from BJAB cells stably expressing EBNA1 and vector only as control for RT-PCR. The analyses showed that EBNA1 enhanced the survivin transcript levels to 3.5-fold and 4.2-fold in two Burkitt's lymphoma lines BL41 and BJAB, respectively (Figs. 2A and B). These results were also consistent with the gene array data. Furthermore, the effect of EBNA1 on the protein level of survivin was analyzed by Western blot. Similarly, survivin levels were also dramatically up-regulated when EBNA1 was expressed (Fig. 2C). These results support the contribution of EBNA1 to up-regulation of survivin.

EBNA1 up-regulates the survivin promoter activity in a dose-dependent manner

Since EBV-infected and EBNA1 expressing cells showed higher levels of survivin mRNA and protein levels, we further explored the molecular mechanism of survivin promoter up-regulation by EBNA1. To analyze the role of EBNA1 on regulating the survivin promoter, we cloned the full-length promoter into pGL3B vector (Lu et al., 2009). A fixed amount of full-length survivin promoter-driving luciferase was cotransfected with increasing doses of pA3M-EBNA1 and total DNA amount was normalized by the pA3M vector into DG75 and 293 cells. After 24 h, the cells were harvested for luciferase reporter assays. The reporter assays showed that EBNA1 can indeed activate the survivin promoter by approximately 3- to 6-fold compared to that of the vector alone in DG75 cells as well as in 293 cells in a dose-dependent fashion (Figs. 3A and B). These results suggest that EBNA1 can up-regulate survivin expression through regulation of cis-acting elements in the promoter.

Cis-acting elements within the survivin promoter are important for EBNA mediated induction of survivin expression

To further confirm that survivin promoter activation is due to EBNA1 expression, we defined the cis-elements responsible for the activation of survivin transcription by EBNA1 through 5′-deletion mutants of the survivin promoter generated by restriction-enzyme digestion cloned upstream of a luciferase reporter. These constructs were transfected into HEK 293 cells in the presence or absence of EBNA1 expression vector. Luciferase activity was measured and representative results are shown in Fig. 4.

The results showed that a deletion of the NK2 transcription factor related, locus 5 (Nkx2.5), a cardiac homeobox transcription factor, at the 5′-end of the survivin promoter (SurPΔNkx) resulted in a more dramatic effect on survivin promoter activity with EBNA1 of approximately 9-fold compared to the full-length survivin promoter (SurP) of about 2-fold and suggest that this element may contribute to EBNA1 mediated up-regulation. However, when the NKx 2.5 and Sp1 sites were both deleted (SurP −269/+1) the fold change was similar

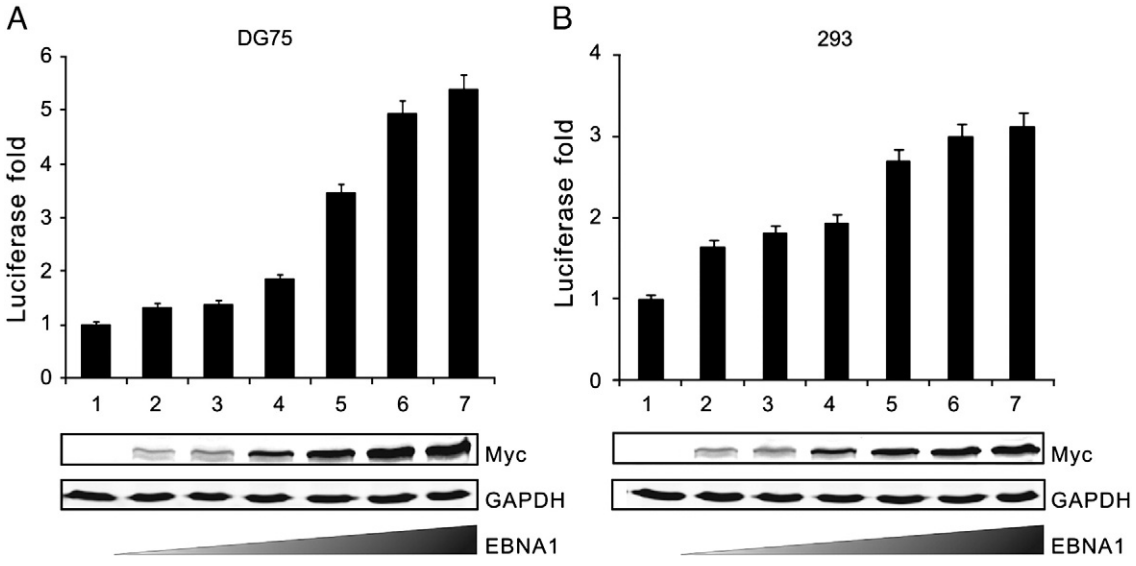


Fig. 3. EBNA1 activated the survivin promoter in a dose-dependent manner. A fixed amount (2 μg) of the reporter plasmids was transfected or cotransfected with pA3M-EBNA1 (1, 2, 5, 10, 15 and 20 μg [numbers 1 to 6]) into 10 million (A) DG75 cells and (B) 293 cells, individually. At 24 h posttransfection, cell lysate of each transfection was harvested for the luciferase assay. The promoter activity was presented as the fold activation relative to the reporter-alone control. Definitely, EBNA can activate the survivin promoter by approximately 3 to 6-fold compared to that of the vector control in DG75 cells as well as in 293 cells in dose-dependent manner. The means and standard deviations from three independent experiments were shown. Western blots showed the expression of myc-tagged EBNA1 in cotransfected cells. The blot was stripped and reprobed with anti-GAPDH antibody to show equal protein loading.

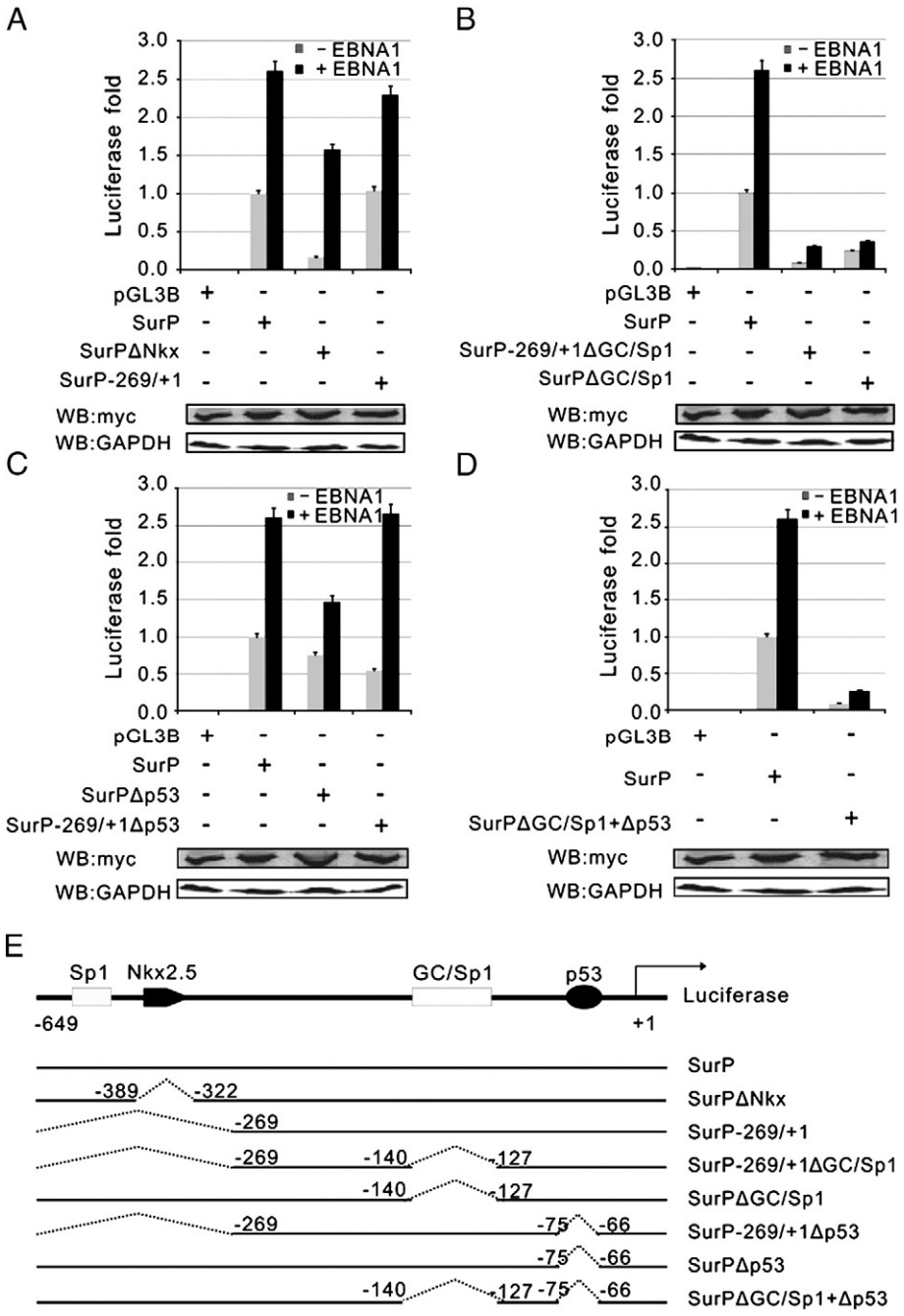


Fig. 4. Cis-acting elements within the survivin promoter are important for EBNA1 mediated induction of survivin expression. (A to D) A fixed amount of the wild type (WT) or mutation type (MT) of survivin promoter reporter plasmid was transfected or cotransfected into 10 million 293 cells with pA3M-EBNA1 expression vector. At 24 h posttransfection, cell lysate of each transfection was harvested for a luciferase assay. Western blots show the expression of EBNA1 in the cotransfected cells. The deletion of GC/Sp1 box (SurPΔGC/Sp1) in the survivin promoter resulted in an almost complete loss of promoter activity in the presence of EBNA1. The blot was stripped and reprobed with anti-GAPDH antibody to show equal protein loading. +, presence; −, absence. (E) Schematic showing the survivin WT and MT promoter used in this study. The putative domains include an NKx 2.5, Sp1 binding sites, GC/Sp1 domain and p53 conserved sequence. Numbering is from the initiating ATG.

to that seen with the wild type (SurP) (Figs. 4A and E). The deletion of GC/Sp1 box (SurPΔGC/Sp1) of the survivin promoter resulted in an almost complete loss of promoter activity (Figs. 4B and E). Further, mutation of the p53 responsive element (SurPΔp53) had a small effect on the promoter activity (Figs. 4C and E), whereas deletion of GC/Sp1 box and the p53 responsive element (SurPΔGC/Sp1 + p53) together resulted in little or no change in promoter activity compared to SurPΔGC/Sp1 suggesting that it may not be critical for the p53 site to cooperate with other cis-elements like Sp1 and the GC rich box to regulate survivin expression (Figs. 4D and E). These results suggested that GC/Sp1 was the major cis-element within the survivin promoter responsive to EBNA1 expression as deletion of this region had the most dramatic effect on survivin expression.

The p53 responsive cis-acting element is not responsive to EBNA1 at the survivin promoter

To test whether the p53 cis-acting element was responsive to the EBNA1 expression, similar assays were performed in the p53-null cell line Saos-2. Importantly, mutation of the p53 responsive element had no significant fold change in activity on the survivin promoter when compared to the control pGL3B-SurP (Fig. 5, compare lanes 2 and 7).

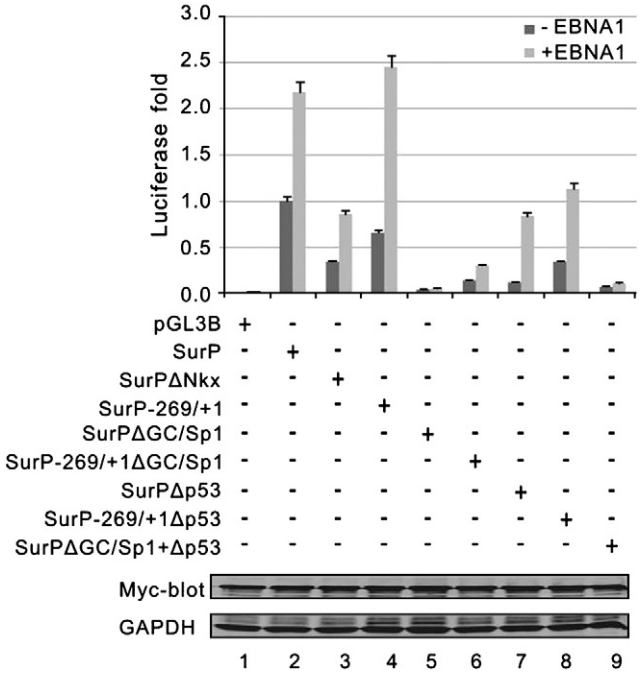


Fig. 5. Reporter assay of the survivin promoter with GC/Sp1 and p53 mutation in Saos-2 cells. A fixed amount of the WT or MT reporter plasmid was transfected into 10 million Saos-2 cells in the presence of pA3M-EBNA1 expression vector. At 24 h posttransfection, cell lysate of each transfection was harvested for a luciferase assay. Obviously, mutation of p53 responsive element had no significant effects on the survivin promoter activity compared to the control pGL3B-SurP with EBNA1. +, presence; −, absence. Western blots show the expression of EBNA1 in cotransfected cells. The blot was stripped and reprobed with anti-GAPDH antibody to show equal protein loading.

However, mutation of the GC/Sp1 site showed a greater effect on the survivin promoter activity compared to the control pGL3B-SurP with and without EBNA1 expression (Fig. 5, compare lane 2 with lanes 5 and 6). In combination, the mutation of the GC/Sp1 and p53 responsive elements resulted in an almost 100% decrease in survivin promoter activity as compared to the control pGL3B-SurP with EBNA1 (Fig. 5, compare lanes 2, 5, and 9). These results suggested that the p53 responsive element in the survivin promoter may not be a major contributor to up-regulation of survivin by EBNA1.

Sp1 forms a complex with EBNA1 and its canonical binding site at the survivin promoter

The survivin gene promoter contains a canonical, Sp1 or Sp1-like element and deletion of these sites results in abrogation of the promoter activity. Thus, the Sp1 transcription factor is involved in survivin gene regulation. To test whether EBNA1 mediated up-regulation of the survivin promoter through formation of a complex with EBNA1 and Sp1 at its cognate sequence, EMSAs were performed. The wild-type and mutant probes for EMSAs were designed to contain Sp1 elements from the survivin promoter and mutations within the Sp1 site where Sp1 would be incapable of binding.

To determine whether EBNA1 formed a complex with Sp1 bound to DNA, double-stranded DNA probes for the Sp1 binding site were labeled and tested for binding to Sp1 protein from the nuclear extracts of Saos-2 cells expressing Sp1. The result showed that the wild-type Sp1 probes had a specific shift with the Sp1 protein from Saos-2 cells (Fig. 6, lane 2). The specificity of the shift was verified by using excess specific cold competitor (Fig. 6, lane 3) which abolished the shift. The use of similar amounts of cold mutant probe was not able to abolish binding of Sp1 to the specific probe (Fig. 6, lane 4) and suggests specificity of the Sp1 interaction at the survivin promoter. That the shift was due to Sp1 was confirmed by using an α-Sp1 specific antibody which supershifted the complex (Fig. 6, lane 5). Further, addition of EBNA1 to the reaction mixture supershifted the entire complex suggesting formation of a complex between Sp1 and EBNA1 (Fig. 6, lane 7). Importantly, the use of anti-myc antibody shifted the complex further confirming the association of the complex of EBNA1, Sp1 and the cis-acting DNA element (Fig. 6, lane 6). Because Sp1 is an essential factor in cells, a shift which is most likely due to endogenous Sp1 is seen in lane 9 (pA3M only). This is confirmed when the cold Sp1 probe is added and the shift disappeared (lane 10). EBNA1 (lane 11) showed a shift compared to a similar Sp1 shift (lane 2). However, when cold Sp1 probe was added, the shift in the EBNA1 lane disappeared suggesting specificity for endogenous Sp1 (lane 12). Together, these results strongly implicated that EBNA1 up-regulates the survivin promoter through formation of a complex with EBNA1 bound to Sp1 at its cognate sequence.

Furthermore, chromatin immunoprecipitation was performed on EBV-positive LCL1 cells. IPs was confirmed by Western blotting (Fig. 6C). Cellular chromatin from LCL1 cells were immunoprecipitated using EBNA1 antibody. Normal mouse IgG was used as a negative control. Recovery of the survivin promoter template was detected by real-time PCR using specific primer amplifying the GC/Sp1 element within the survivin promoter. As shown in Fig. 6D, normal mouse IgG did not amplify any template from survivin promoter, but in the anti-EBNA1 ChIP, similar amounts of survivin promoter sequence were immunoprecipitated with EBNA1 antibody from LCL1 cells compared to input. This supports our result above which demonstrates a specific association of EBNA1 with the survivin promoter (Fig. 6D). This study further demonstrates that EBNA1 binds to Sp1 canonical binding site.

The EBNA1 transactivation domain is responsible for up-regulating the survivin promoter

The data above showed that Sp1 can interact with EBNA1. We therefore decided to determine the domain responsible for up-regulation of survivin expression. Different EBNA1 truncations were cotransfected with the luciferase reporter plasmid carrying the survivin promoter. The promoter activities were monitored by luciferase activities. The mutant p376 which lacks the Gly-Ala repeats and mutants p385 and p396, which lack the looping and DNA binding and a region of the dimerization domain respectively, show a slight decrease in survivin promoter activity compared to wild-type EBNA1 indicating that those domains are important in contribution to EBNA activity at the promoter. The p378 mutant which knocks out the small Gly-Arg repeat region 5′ to the transactivation domain showed a slight stimulus on survivin promoter indicating that this domain is not involved in EBNA1 up-regulation of survivin expression. However, the mutant p367 which truncated the transactivation domain had a significant decrease in its ability to activate the survivin promoter. Therefore the domain which lies between amino acids 65 and 89 is critical for EBNA1 mediated up-regulation of survivin expression (Fig. 7).

Survivin expression is up-regulated in EBV-associated lymphoma tissue

To determine whether the survivin expression is also up-regulated in EBV-associated cancer tissue, sections of EBV-positive and -negative DLBL tissue were stained by immunohistochemistry to determine the presence of EBNA1 and survivin (Fig. 8). Survivin was detected using mouse monoclonal anti-survivin antibody followed by detection using anti-mouse Alexa Fluor 488. EBNA1 was detected using rabbit polyclonal anti-EBNA1 antibody (K67.3) followed by anti-rabbit Alexa Fluor 594. In the immunostained sections, high expression of survivin overlapped with the expression of EBNA1 which showed that in EBV-positive tumor cells survivin expression was also up-regulated. This was clearly distinct from the lack of observed signals in an

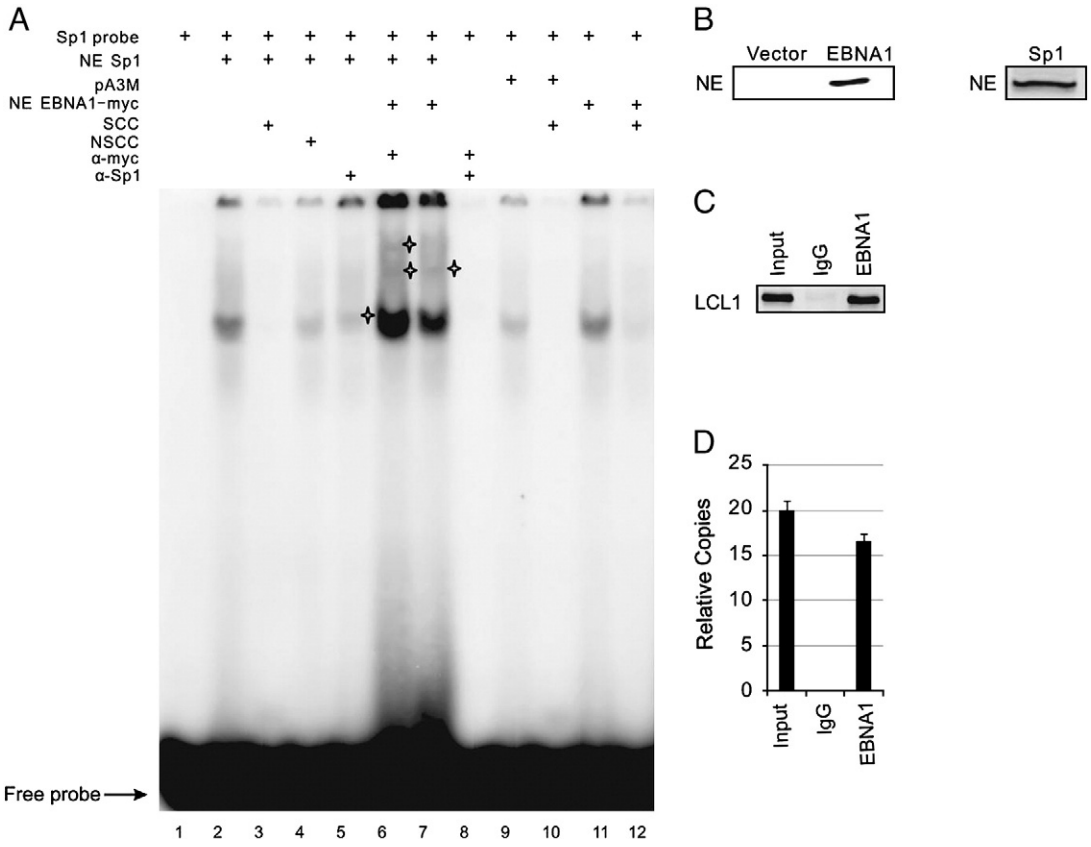


Fig. 6. Sp1 binds to the survivin promoter in complex with EBNA1 at its canonical binding site. Electrophoretic mobility shift assays were performed as described in Materials and methods. (A) Binding of Sp1 site in the survivin promoter with EBNA1 protein as well as Sp1. Lane 1 is probe alone (bottom), lane 2 is probe with nuclear extract containing Sp1 showing faster mobility of the probe which was abolished by adding an excess (200-fold) of unlabeled specific probe (lane 3). The shift in mobility was unaffected with similar amounts (200-fold) of nonspecific unlabeled competitor (lane 4). Adding anti-Sp1 antibody changed the mobility of Sp1 (lane 5, asterisk). Lane 7 contains probe with nuclear extracts of pA3M-EBNA1 showing a change in the mobility of the probe due to the binding of Sp1 to EBNA1 (asterisk). Further, adding anti-myc antibody changed the mobility of the entire complex, supershifting it (lane 6, asterisk). Lane 8, which contains Sp1 and α-myc antibodies with probe, demonstrates that nonspecific binding did not occur. Lane 9 is vector control only but contains the cell essential factor Sp1, This is confirmed when the cold Sp1 probe is added and the shift disappeared (lane 10). The EBNA1 (lane 11) still has some shift compared to Sp1 (lane 2), but when cold Sp1 probe is added, EBNA1 does not show obvious shift compared to EBNA1 (lane 12) NE, nuclear extract. (B) Western blots to detect proteins from nuclear extracts. (C) LCL1 cells were harvested; cross-linked chromatin was precipitated with OT1x antibody or normal mouse IgG. Western blot shows the coimmunoprecipitation using OT1x antibody against EBNA1. (D) The recovered DNA was quantitated by real-time PCR. Shown are the results from three independent experiments. Error bars indicate standard deviations.

EBV-negative tumor tissue section suggesting that EBV infection and EBNA1 expression were important for survivin expression.

Depletion of survivin using shRNA increases the proportion of cells undergoing apoptosis

The cell cycle phases (G1, S or G2/M phase) are characterized by their DNA content. Propidium iodide (PI) binds to DNA at a ratio of 1:1, and hence the DNA content of cell cycle phases is a reflection of their PI fluorescent intensities. Cell cycle analysis was performed on a FACSCalibur cytometer. We hypothesized that EBNA1 can usurp the apoptotic pathway to regulate cellular apoptosis through regulation of survivin expression. Transduction and selection of EBV-infected cells by lentivirus-delivered shRNA resulted in stable cell lines expressing survivin-specific shRNA and EBNA1 specific shRNA as well as a control shRNA vector. The cells with GFP immunofluorescence were monitored using an Olympus IX71 fluorescent microscope (Fig. 9A). The expression levels of survivin and EBNA1 in these cells along with control shRNA were determined by Western blot analysis (Fig. 9B). The results showed that survivin and EBNA1 expression levels were knocked down in cells infected with survivin or EBNA1 specific shRNA lentivirus (Fig. 9B). Furthermore, analysis of cells for apoptosis showed that the LCL1 cells infected with the sh-SV lentivirus showed an increase in cell apoptosis compared to the LCL1 infected sh-C cells (Fig. 9C). LCL1 cells with a lentivirus-delivered shRNA that specifically knocks down EBNA1 expression in these cells (LCL1-sh-E1) led to a decrease in apoptosis compared to LCL-sh-SV (Fig. 9C) albeit less than seen directly with sh-SV lentivirus. This was expected as the level of survivin shutdown was not as efficient compared to when survivin was directly targeted by sh-SV. The results support a critical role for survivin in viral infected cells with a direct role in suppression of apoptosis in the EBV-infected lymphoma cells contributing to the cell survival.

Discussion

Many tumor viruses play an essential role in both the initiation and subsequent progression of cancer. Survivin is an inhibitor of the apoptosis protein expressed in many human cancer cells but not in most normal adult tissues (Ambrosini et al., 1997). Its expression is up-regulated by a number of proteins encoded by tumor viruses (Lu et al., 2009; Pina-Oviedo et al., 2007; Zhang et al., 2005; Zhou and Gong, 2006; Zhu et al., 2003). The E6 protein of human papillomavirus type 16 up-regulates survivin expression through interaction with p53 (Borbely et al., 2006). The survivin promoter was also transactivated by the T-cell leukemia virus type I Tax protein, via the activation of NF-κB (Kawakami et al., 2005). Survivin is also up-regulated by the hepatitis B virus X protein (HBx), forms a complex with HBx-interacting protein and binds pro-caspase 9, preventing its recruitment by Apaf1 in hepatoma cells (Marusawa et al., 2003). Recently,

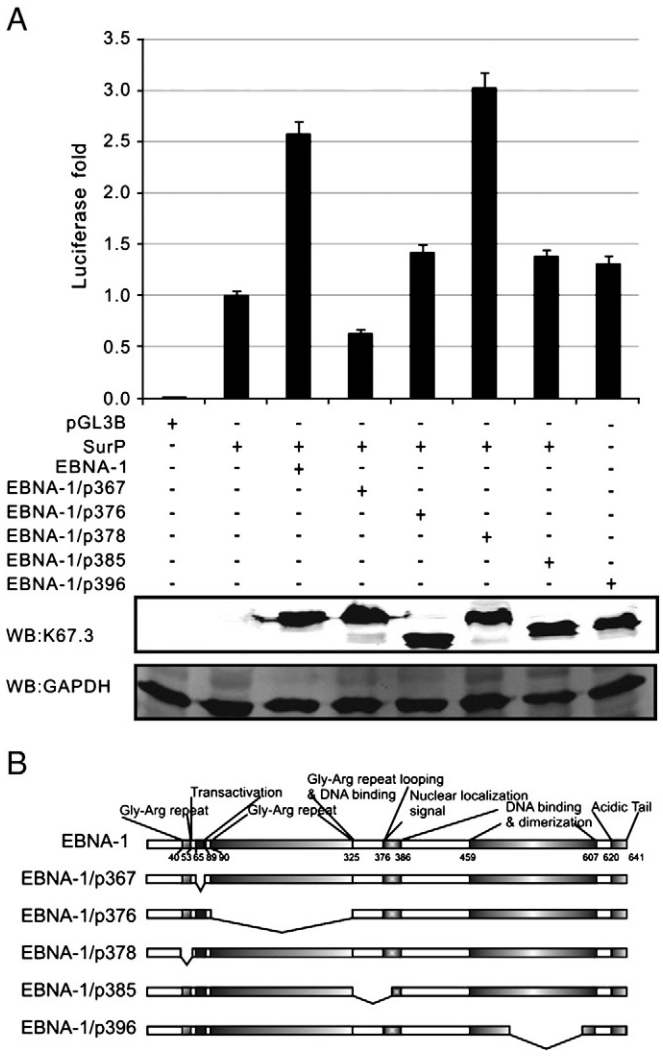


Fig. 7. The EBNA1 transactivation domain is required for activation of the survivin promoter. (A) A fixed amount of the WT or MT EBNA1 expression vector was transfected into 10 million 293 cells in the presence of survivin promoter reporter plasmid. At 24 h posttransfection, cell lysate of each transfection was harvested for a luciferase assay. Results of luciferase assay showed that EBNA1/p367 had a significant decrease in its ability to activate the survivin promoter. All the transfections were done multiple times, and the results shown represent the means of the data from three independent experiments. Western blots show the expression of EBNA1 and truncations in the cotransfected cells. The blot was stripped and reprobed with anti-GAPDH antibody to show equal protein loading. +, presence; −, absence. (B) Schematic showing the EBNA1 truncation used in this study.

the EBV encoded LMP2A protein was shown to up-regulate survivin expression in EBV-associated gastric carcinoma (Hino et al., 2008). Therefore up-regulation of survivin may be a common denominator in viral mediated oncogenesis. Additionally, EBNA-1 mRNA levels are up-regulated in S phase (Davenport and Pagano, 1999). Thus EBNA1 may provide (survivin and/or USP7/p53-related) survival effects in dividing cells countering signals that instruct the cell to stop and repair damage. Recently, Frappier L. et al. reported that EBNA1 affects PML bodies and potentially affects DNA repair also supporting the pro-oncogenic potential of EBNA1 (Sivachandran et al., 2008). This may be relevant for "general" survival of dividing EBV-positive cells in normal biology, and contributes to the oncogenic risk in EBV-positive cells that are challenged or contain damaged DNA.

EBV is a ubiquitous human herpesvirus associated with the development of both lymphoid and epithelial tumors (Rickinson and Kieff, 2001). The pattern of EBV latent protein expression in these tumors is different from that observed in EBV-transformed lymphoblastoid cell lines (LCLs) (Raab-Traub, 2002; Young and Rickinson, 2004). Thus, only EBNA1 is typically expressed in Burkitt's lymphoma (BL), while EBNA1 and two membrane proteins (LMP1 and LMP2A/B) are expressed in Hodgkin's lymphoma (HL) and nasopharyngeal carcinoma (NPC) (Raab-Traub, 2002; Rickinson and Kieff, 2001). However, a more direct involvement in oncogenesis has been suggested by the ability of B-cell-directed EBNA1 expression to produce B-cell lymphomas in transgenic mice, although these are still unresolved questions (Kang et al., 2005; Wilson et al., 1996), as to its possible contribution to BL cell survival in vitro (Kennedy et al., 2003).

In this report we showed that EBNA1, which is known to be expressed in all EBV-positive tumors, up-regulated survivin expression in the EBV-infected B-lymphoma cells. Survivin was shown to be up-regulated in human cancers showing its antiapoptotic activity and its ability to regulate cell-cycle progression (Ambrosini et al., 1997). Importantly, EBNA1 is expressed in all forms of EBV-infected cells and plays a role in segregation and maintenance of the episomal EBV genome (Imai et al., 1998; Rickinson and Kieff, 1996, 2001). EBNA1 is consistently detected in all EBV-associated tumors, including lymphoproliferative disease (Hammerschmidt and Sugden, 2004). The function of EBNA1 has been extensively studied in B cells, where in vitro infection with EBV is efficient and results in B-cell transformation (Rickinson and Kieff, 1996; Yates et al., 1985). In addition, increasing evidence also suggests that EBNA1 may directly contribute to tumorigenesis by inhibiting apoptosis (Kennedy et al., 2003; Saridakis et al., 2005). However, its role with regard to survivin in EBV-infected B-lymphoma cells is yet to be completely elucidated.

The possibility that EBNA1 has additional functional effects beyond its crucial role in the maintenance and replication of the EBV episome has been suggested for some time. The ability of EBNA1 to transcriptionally regulate viral genes is well accepted and previous reports implicated EBNA1 in the control of specific cellular genes in B cells (Tsimbouri et al., 2002). Moreover, Wood et al. (2007) reported that EBNA1 regulates cellular gene transcription and modulates STAT1 and TGFβ signaling pathways. Our gene array analysis from BJAB expressing EBNA1 showed that many cellular factors were up-regulated in EBNA expressing cells, including one referred to as the inhibitor of apoptosis protein also called survivin. This suggests that EBNA1 can activate survivin gene expression. Up-regulation of survivin expression was due to LMP2A in EBV-associated gastric carcinoma (Hino et al., 2008). However, EBV-associated lymphoma cells do not express all the latent proteins, leading to restricted forms of latency in which EBNA1 is detected alone (Latency I, found in BL). We further decided to investigate a potential mechanism linked to development of EBV-associated cancer through an increasing survivin expression by the major latent protein EBNA1 in the EBV-infected B-lymphoma.

The expression of survivin was highly induced with the expression of EBNA1 seen in the immunostained sections which suggested that EBV-positive cells up-regulated survivin expression in tumor cells. This indicated that EBNA1, in addition to other latent proteins, may play an important role in up-regulation of survivin expression in EBV-associated lymphoma, thus contributing to EBV-associated tumorigenesis. However, the molecular basis for the observed effects of EBNA1 on cellular apoptosis is largely unknown, though expression of EBNA1 in U2OS cells was shown to protect these cells from apoptosis in response to DNA damage by interfering with p53 stabilization (Saridakis et al., 2005). In a recent study, TE7 cells showed loss of p53 expression and demonstrated increased transcription of the survivin gene. Overexpression of wild-type human p53 in TE7 cells decreased transcription of the survivin gene as determined by the mRNA level (Chang et al., 2010). Here we confirmed that EBNA1 can form a complex with Sp1 bound to its responsive element on the survivin promoter and so up-regulate survivin expression. However, up-regulation of survivin is not likely due to a complex of EBNA1 and p53 bound to its binding site. These

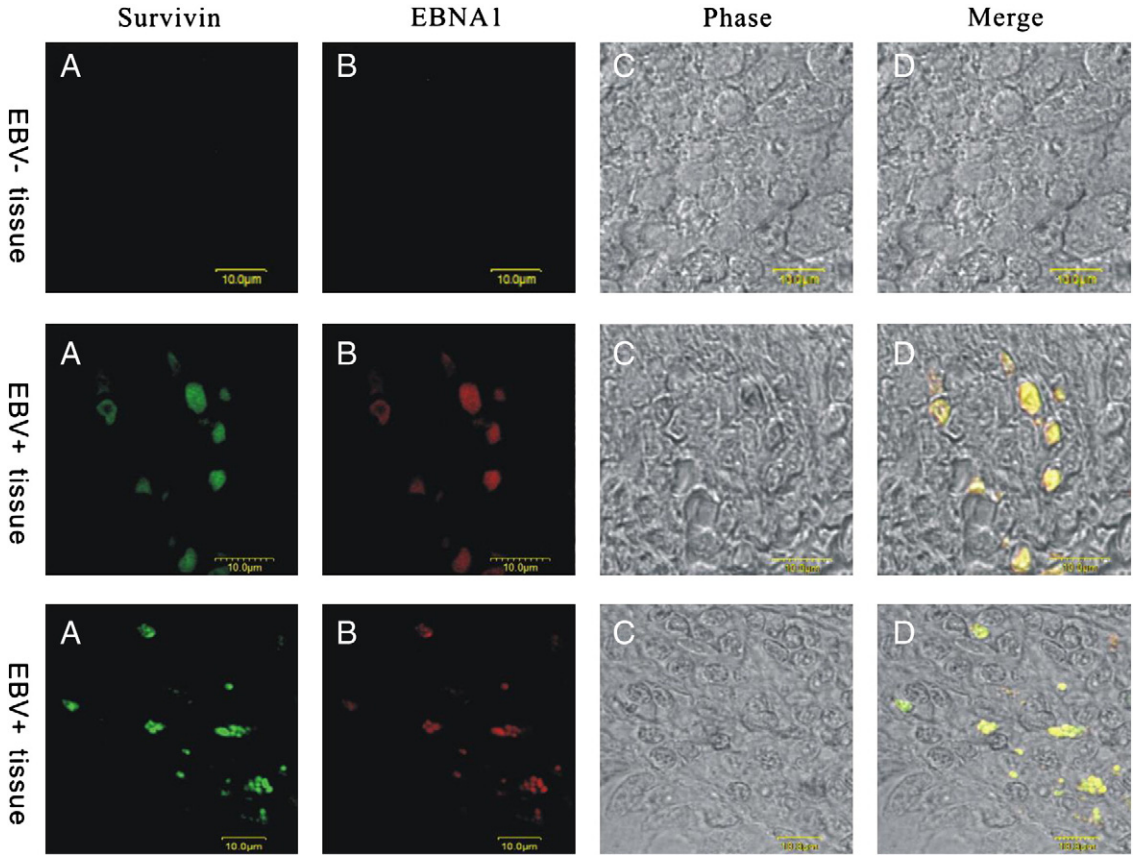


Fig. 8. Survivin expression is up-regulated in EBV-associated lymphoma tissue. Immunohistochemistry assays for survivin and EBNA1 were performed in EBV-positive and -negative tissue specimens. Paraffin-embedded EBV-positive and -negative diffuse large B-cell lymphoma (DLBL) tissues were deparaffinized, rehydrated, and washed with phosphate buffered saline. Following antigen retrieval, samples were blocked with BSA prior to incubation with primary antibodies overnight at 4 °C. Survivin was detected by using mouse monoclonal anti-survivin antibody followed by detection using anti-mouse Alexa Fluor 488 (panel A) and EBNA1 was detected by using rabbit polyclonal anti-EBNA1 antibody K67.3 followed by anti-rabbit Alexa Fluor 594 (panel B). The Merge panel shows most of the survivin co-expression with EBNA1 (panel D).

results are consistent with previous reports which showed that when Sp1 and EBNA1 were cotransfected with the reporter plasmid, the cooperative effects of Sp1 and EBNA1 resulted in an approximately 2-fold increase in Cp activation compared to the effect of Sp1 alone (Nilsson et al., 2001). Reporter assays using the truncations of EBNA1 protein showed that the EBNA1 transactivation domain is an important region required for EBNA1 binding to Sp1 with the survivin promoter resulting in up-regulation of survivin expression. Knock down of EBNA1 expression in the EBV-associated cells further supported our hypothesis that EBNA1 can up-regulate survivin, enhance resistance of cell to apoptosis and so increase cell survival (Fig. 9D). These findings strongly suggest a role for EBNA1 in suppressing of cell apoptosis and may thus contribute to the multistep process of tumor development. Here the results suggest that EBV encoded EBNA1 can support the oncogenic process by up-regulating the apoptosis suppressor protein survivin in EBV-associated B-lymphoma cells which leads to tumor development and provides further insights into the overall contributions of EBV to human cancer.

Materials and methods

Constructs, cell lines and antibodies

The expression constructs of EBNA1 (pcDNA-EBNA1) used in the present study have been described earlier (Murakami et al., 2005). Mutant EBNA1 p367, p376, p378, p385, and p396 were kindly provided by John Yates (1988).

The EBV-negative cell lines BJAB and DG75 and the EBV-positive cells LCL1 were cultured in an RPMI 1640 medium supplemented with 7% fetal bovine serum, 2 mM L-glutamine, and penicillin–streptomycin (5 U/ml and 5 μg/ml, respectively). The human embryonic kidney 293 (HEK293) cell line and p53-null Saos-2 osteosarcoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum, 2 mM L-glutamine, and penicillin–streptomycin (5 U/ml and 5 μg/ml, respectively). BJAB cells stably expressing EBNA1 were described previously (Kaul et al., 2007). BL41–B958 cell line is an EBV-infected Burkitt's lymphoma cell line and has been previously described (Subramanian et al., 2001).

Survivin antibody was purchased from Cell Signaling Technology, Inc. (Danvers, MA). Rabbit polyclonal antibody K67.3 and mouse monoclonal antibody OT1x were kindly supplied by Jaap Middeldorp (Department of Pathology at VU University Medical Center, Amsterdam, The Netherlands) (Sears et al., 2004). The rabbit serum against EBNA1 (raised against EBNA1-[452–641]) was kindly supplied by Lori Frappier (Department of Molecular Genetics at the University of Toronto, Ontario, Canada) (Holowaty et al., 2003). Antibody for Sp1 transcription factor was purchased from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA).

RNA extraction, gene array hybridization and data analysis

Total RNA was extracted from BJAB expressing EBNA1 or BJAB-vector cells using TRIzol reagent according to the manufacturer's instruction (Life Technologies, Gaithersburg, MD). Gene arrays were performed essentially as described previously (Lu et al., 2009) using the super array cell-cycle membrane (OHS-020). Data acquisition and quantification of spot intensities were performed by using the

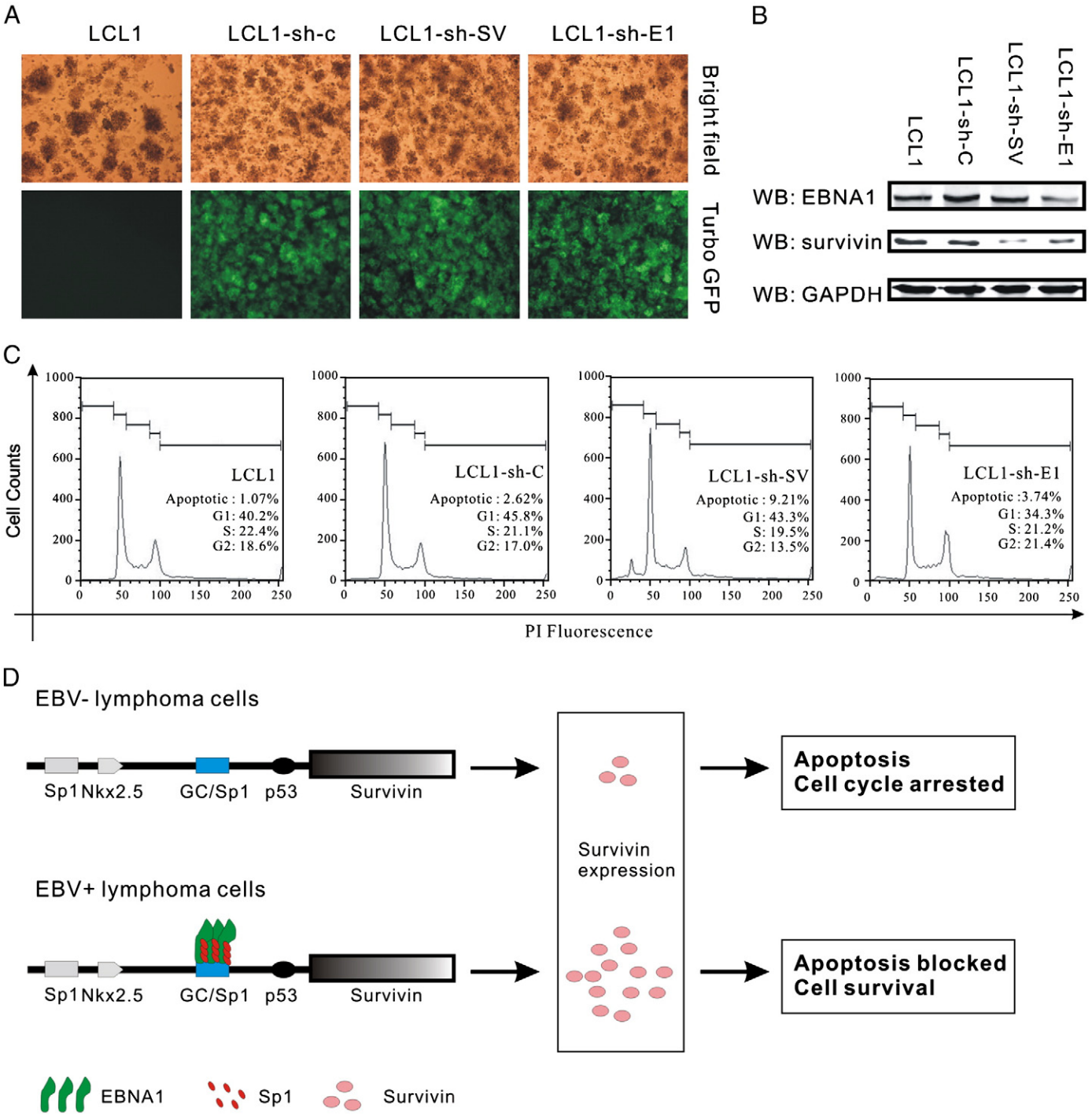


Fig. 9. Depletion of survivin by shRNA increases the proportion of cell undergoing apoptosis. (A) LCL1 cells were infected with a lentivirus encoding survivin shRNA and analyzed by fluorescence microscopy after 48 h for the expression of GFP. (B) Western blotting showing expression of EBNA 1 and survivin in EBV non-infected cell line and EBV-infected cell line. GAPDH level was used for internal control. (C) Flow cytometry analysis of different treated cells. LCL1 cells were treated with survivin shRNA and then washed, fixed, stained with propidium iodide (PI), and analyzed for their DNA content by flow cytometry. LCl1 cells infected with the sh-SV lentivirus had an increase in cell apoptosis compared to the LCL1 infected sh-C cells, LCL1 cells with a lentivirus-delivered shRNA that knocks down EBNA1 expression in these cells (LCL1-sh-E1) led to a decrease in apoptosis compared to LCL1-sh-SV. sh-SV, survivin shRNA; sh-C, control shRNA; sh-E1, EBNA1 shRNA. (D) Hypothetical model showing that EBV encoded EBNA1 contributes to up-regulation of survivin and resistance to apoptosis in EBV-associated B-lymphoma cells.

GEArray Expression Analysis suite software 2.0 (SABiosciences, Frederick, MD).

Immunofluorescence

Immunofluorescence assays were performed essentially as described previously (Lu et al., 2009). Briefly, BJAB-vector and BJAB-EBNA1 stable cells on cover slides were fixed in 3% paraformaldehyde with 0.1% Triton X-100 for 20 min. Cover slides were washed with 1× PBS and subsequently blocked in 1% bovine serum albumin for 10 min. Cells were washed with 1× PBS and EBNA1 were detected using rabbit serum raised against EBNA1 (Holowaty et al., 2003) followed by detection using anti-rabbit Alexa Fluor 488 (Molecular Probes, Eugene, OR). The slides were examined with a Fluoview FV300 (Olympus, Melville, NY) confocal

microscope and the images were analyzed with FLUOVIEW software (Olympus, Melville, NY).

Luciferase assay

Luciferase assays were performed as described previously (Lu et al., 2009). Luminescence was measured for 10 s by the Opticomp I luminometer (MGM Instruments, Hamden, CT). The lysates were also tested at various dilutions to ensure that luciferase activity was within the linear range of the assay. The results shown represent experiments performed in triplicate.

Real-time quantitative PCR

Total RNAs from cells were collected using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. cDNA was made using high capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. The specific primers for survivin are 5′-CAGCCCTTTCTCAAGGACCA-3′ and 5′-TGTTCCTCTATGGG GTCGTC-3′; for GAPDH (glyceraldehyde-3-phosphate dehydrogenase), 5′-TGCACCACCAACTGCTTAG-3′ and 5′-GATGCAGGGATGATGTTC-3′. The cDNA was amplified using Power SYBR green PCR master mix (Applied Biosystems, Foster City, CA), 1 μM each primer, and 1 μl of the cDNA product in a total volume of 20 μl. Thirty-five cycles of PCR (1 cycle consisting of 1 min at 94 °C, 30 s at 56 °C, and 40 s at 72 °C), followed by 7 min at 72 °C, were performed in an MJ Research Opticon II thermocycler (MJ Research, Waltham, MA). Each cycle was followed by two plate readings, with the first at 72 °C and the second at 85 °C. A melting curve analysis was performed to verify the specificity of the products, and the values for the relative quantitation were calculated by the $\Delta\Delta C_t$ method. The experiment was performed in triplicate.

Preparation of nuclear extracts and electrophoretic mobility shift assay

Nuclear extracts were prepared from transiently transfected Soas-2 cells with different expression constructs and an empty vector as described previously (Verma and Robertson, 2003). Specific probes were used for the electrophoretic mobility shift assay (EMSA) encompassing the GC/Sp1 box (5′-GCACCCCGCGCCGCCCCGCCTC TACTCCCAG-3′) and mutant GC/Sp1 box (5′-GCACCCGAATTCATG-GATCCTCTACTCCCAG-3′). The ^{32}P-labeled probes were synthesized via the Klenow fill-in reaction and purified with Select-D G-25 columns (Shelton/IBI, Peosta, IA). Radioactive probes were diluted in TE buffer to a final concentration of 80,000 cpm/μl. DNA-binding reactions were performed in a manner similar to that described previously (Knight et al., 2001). Proteins from nuclear extracts were mixed with 1 μg of poly (dC/dI) (Sigma-Aldrich, St. Louis, MO) in 1× DNA-binding buffer for 5 min at room temperature; 1 μl of labeled probe was added to each reaction mixture, and the tubes were incubated at room temperature for 15 min. DNA–protein complexes were resolved in a nondenaturing 6% polyacrylamide gel electrophoresis. The gel was run in 0.5× Tris–borate–EDTA buffer at a constant voltage of 120 V for 4 h. Following electrophoresis, the gel was transferred to Whatman paper and dried for 1 h at 80 °C. Dried gels were exposed to a phosphorimager screen for 12 h (Amersham Biosciences, Piscataway, NJ) and scanned by a PhosphorImager (Molecular Dynamics, Piscataway, NJ). For specificity determination, cold competitor probe was added at a 200-fold molar excess. For supershift experiments, polyclonal antibodies against Sp1 were added after an initial 30-min incubation of probe with nuclear extract and incubated for 1 h at 4 °C.

Immunohistochemistry

Slides mounted with sections of paraffin-embedded, archival, deidentified EBV-positive and -negative Diffuse large B-cell lymphoma (DLBL) tissue specimens were a generous gift from Mariusz A. Wasik (Department of Pathology and Laboratory Medicine, Hospital of the University of Pennsylvania, Philadelphia, PA). Slides were deparaffinized, rehydrated, and washed with PBS. Following antigen retrieval in 0.5%:5 mg/ml pepsin with 0.1 N HCL at 37 °C for 10–15 min, samples were blocked with 2% fish stain gelatin prior to incubation with primary antibodies overnight at 4 °C. Survivin was detected by using mouse monoclonal anti-survivin antibody followed by anti-mouse Alexa Fluor 488 (Molecular Probes, Eugene, OR), and EBNA1 was detected by using Rabbit polyclonal antibody K67.3 followed by anti-rabbit Alexa Fluor 594 (Molecular Probes, Eugene, OR). Fluorescence confocal microscopy was performed with an Olympus microscope (Olympus, Melville, NY).

Construction of lentiviral vectors

The small hairpin RNAs (shRNA) against EBNA1 (GGAGGTTCCA ACCCGAAAT) were constructed by annealing two primers containing the 19-nt sense and reverse complementary targeting sequences with a 9-nucleotide loop – TCTCTTGAA – and flanking XhoI and MluI cloning sites and then cloned into pGIPZ (Open Biosystems, Huntsville, AL) plasmid (EBNA1 shRNA is hereinafter abbreviated as sh-E1) (Yin and Flemington, 2006). The sequences of the insertions were confirmed by DNA sequencing. The control shRNA (abbreviated as sh-C) and survivin shRNA (abbreviated as sh-SV) were described previously (Lu et al., 2009).

Lentivirus production and establishment of shRNA expressing stable cell lines

Lentivirus with shRNA expression cassette was produced by calcium phosphate-mediated, three-plasmid transfection of 293 T cells as previously described (Lu et al., 2009). Recombinant lentiviruses were concentrated by spinning at 23,500 rpm for 2.5 h, then dissolved in RMPI 1640 (HyClone, Logan, UT) at room temperature for 30 min. Titers were determined by infecting 293 T cells with serial dilutions of concentrated lentivirus and counting Turbo GFP positive cells after 48 h under fluorescent microscopy. The concentrated virus was used to infect 10^5 cells in a six-well plate in the presence of Polybrene (8 mg/ml). After 72 h, the medium was replaced with 2 ml RPMI 1640 containing 2 μg/ml puromycin. For selection, colonies were expanded and expression checked by Western blot analysis. The control shRNA were labeled as sh-C and survivin shRNA expressing cells were labeled as sh-SV.

Flow cytometry and apoptosis assays

These assays were performed essentially as described previously (Riccardi and Nicoletti, 2006). Briefly, cells were harvested and fixed in 70% ice-cold ethanol and stored at −20 °C for later analysis. The fixed cells were collected by centrifugation at 400 *g* and washed with cold PBS–saline twice. Cells were suspended in propidium iodide (PI) staining solution (50 μg/ml final concentration) supplemented with 200 μg of RNase A per ml and incubated for 30 min at room temperature and then overnight at 4 °C in the dark. Cell-cycle distribution was then analyzed by flow cytometry with a FACScan instrument (Becton–Dickinson, Franklin Lakes, NJ). Total populations were gated to remove doublets and small debris. A total of 20,000 events were analyzed for each sample. Data was analyzed using the ModFIT model program (Becton–Dickinson, Franklin Lakes, NJ).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) was performed as previously described (Verma et al., 2006, 2007). Briefly, EBV-positive cells LCL1 were collected and DNA was cross-linked and then sheared by sonication to an average length of 700 bp, as confirmed by agarose gel electrophoresis. Quantification of recovered DNA was determined using real-time PCR (StepOnePlus™ Real-Time PCR Systems; Applied

Biosystems, Foster City, CA) and primers (For GC/sp1 responsive element, 5′ AAAGCAGTCGAGGGGGCG 3′ and 5′ TTCTGGGAGTAGAGG CGG 3′) designed with computer software (DNASTAR Lasergene 8, Madison, WI). The entire experiment was performed in triplicate and error bars indicate standard deviations.

Acknowledgments

We thank Dario C. Altieri (Department of Cancer Biology, University of Massachusetts Medical School, Worcester, MA) for the pLuc-649c plasmid with full-length survivin promoter, Dr. Lori Frappier (Department of Molecular Genetics, University of Toronto, Ontario, Canada) for rabbit serum raised against EBNA1 and Dr. Jaap Middeldorp for rabbit polyclonal antibody K67.3 and mouse monoclonal antibody OT1x (Department of Pathology at VU University Medical Center, Amsterdam, The Netherlands). We also thank Mariusz A. Wasik (Department of Pathology and Laboratory Medicine, Hospital of the University of Pennsylvania, Philadelphia, PA) for EBV tissue slides. This work was supported by public health service grants NCI CA072510 and CA091792 and NIDCR DE017338 (to E.S.R.). E.S.R. is a scholar of the Leukemia and Lymphoma Society of America. S.C.V is supported by an NIH pathway to independence award (K99CA126182).

References

Altieri, D.C., 2003. Validating survivin as a cancer therapeutic target. Nat. Rev. Cancer 3 (1), 46–54.
Altieri, D.C., 2008. New wirings in the survivin networks. Oncogene 27 (48), 6276–6284.
Ambrosini, G., Adida, C., Altieri, D.C., 1997. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nat. Med. 3 (8), 917–921.
Boidot, R., Vegran, F., Jacob, D., Chevrier, S., Cadouot, M., Feron, O., Solary, E., Lizard-Nacol, S., 2010. The transcription factor GATA-1 is overexpressed in breast carcinomas and contributes to survivin upregulation via a promoter polymorphism. Oncogene 29 (17), 2577–2584.
Borbely, A.A., Murvai, M., Konya, J., Beck, Z., Gergely, L., Li, F., Veress, G., 2006. Effects of human papillomavirus type 16 oncoproteins on survivin gene expression. J. Gen. Virol. 87 (Pt 2), 287–294.
Canaan, A., Haviv, I., Urban, A.E., Schulz, V.P., Hartman, S., Zhang, Z., Palejev, D., Deisseroth, A.B., Lacy, J., Snyder, M., Gerstein, M., Weissman, S.M., 2009. EBNA1 regulates cellular gene expression by binding cellular promoters. Proc. Natl Acad. Sci. USA 106 (52), 22421–22426.
Chang, E., Donahue, J., Smith, A., Hornick, J., Rao, J.N., Wang, J.Y., Battafarano, R.J., 2010. Loss of p53, rather than beta-catenin overexpression, induces survivin-mediated resistance to apoptosis in an esophageal cancer cell line. J. Thorac. Cardiovasc. Surg. 140 (1), 225–232.
Chen, J.S., Liu, J.C., Shen, L., Rau, K.M., Kuo, H.P., Li, Y.M., Shi, D., Lee, Y.C., Chang, K.J., Hung, M.C., 2004. Cancer-specific activation of the survivin promoter and its potential use in gene therapy. Cancer Gene Ther. 11 (11), 740–747.
Cheng, T.C., Hsieh, S.S., Hsu, W.L., Chen, Y.F., Ho, H.H., Sheu, L.F., 2010. Expression of Epstein–Barr nuclear antigen 1 in gastric carcinoma cells is associated with enhanced tumorigenicity and reduced cisplatin sensitivity. Int. J. Oncol. 36 (1), 151–160.
Chun, J.Y., Hu, Y., Pinder, E., Wu, J., Li, F., Gao, A.C., 2007. Selenium inhibition of survivin expression by preventing Sp1 binding to its promoter. Mol. Cancer Ther. 6 (9), 2572–2580.
Davenport, M.G., Pagano, J.S., 1999. Expression of EBNA-1 mRNA is regulated by cell cycle during Epstein–Barr virus type I latency. J. Virol. 73 (4), 3154–3161.
Fodde, R., Brabletz, T., 2007. Wnt/beta-catenin signaling in cancer stemness and malignant behavior. Curr. Opin. Cell Biol. 19 (2), 150–158.
Gahn, T.A., Sugden, B., 1995. An EBNA-1-dependent enhancer acts from a distance of 10 kilobase pairs to increase expression of the Epstein–Barr virus LMP gene. J. Virol. 69 (4), 2633–2636.
Gruhne, B., Sompallae, R., Marescotti, D., Kamranvar, S.A., Gastaldello, S., Masucci, M.G., 2009. The Epstein–Barr virus nuclear antigen-1 promotes genomic instability via induction of reactive oxygen species. Proc. Natl Acad. Sci. USA 106 (7), 2313–2318.
Hammerschmidt, W., Sugden, B., 2004. Epstein–Barr virus sustains Burkitt's lymphomas and Hodgkin's disease. Trends Mol. Med. 10 (7), 331–336.
Hino, R., Uozaki, H., Inoue, Y., Shintani, Y., Ushiku, T., Sakatani, T., Takada, K., Fukayama, M., 2008. Survival advantage of EBV-associated gastric carcinoma: survivin up-regulation by viral latent membrane protein 2A. Cancer Res. 68 (5), 1427–1435.
Holowaty, M.N., Zeghouf, M., Wu, H., Tellam, J., Athanasopoulos, V., Greenblatt, J., Frappier, L., 2003. Protein profiling with Epstein–Barr nuclear antigen-1 reveals an interaction with the herpesvirus-associated ubiquitin-specific protease HAUSP/USP7. J. Biol. Chem. 278 (32), 29987–29994.
Hong, M., Murai, Y., Kutsuna, T., Takahashi, H., Nomoto, K., Cheng, C.M., Ishizawa, S., Zhao, Q.L., Ogawa, R., Harmon, B.V., Tsuneyama, K., Takano, Y., 2006. Suppression of Epstein–Barr nuclear antigen 1 (EBNA1) by RNA interference inhibits proliferation of EBV-positive Burkitt's lymphoma cells. J. Cancer Res. Clin. Oncol. 132 (1), 1–8.
Hung, S.C., Kang, M.S., Kieff, E., 2001. Maintenance of Epstein–Barr virus (EBV) oriP-based episomes requires EBV-encoded nuclear antigen-1 chromosome-binding domains, which can be replaced by high-mobility group-I or histone H1. Proc. Natl Acad. Sci. USA 98 (4), 1865–1870.
Imai, S., Nishikawa, J., Takada, K., 1998. Cell-to-cell contact as an efficient mode of Epstein–Barr virus infection of diverse human epithelial cells. J. Virol. 72 (5), 4371–4378.
Kang, M.S., Lu, H., Yasui, T., Sharpe, A., Warren, H., Cahir-McFarland, E., Bronson, R., Hung, S.C., Kieff, E., 2005. Epstein–Barr virus nuclear antigen 1 does not induce lymphoma in transgenic FVB mice. Proc. Natl Acad. Sci. USA 102 (3), 820–825.
Kapoor, P., Lavoie, B.D., Frappier, L., 2005. EBP2 plays a key role in Epstein–Barr virus mitotic segregation and is regulated by aurora family kinases. Mol. Cell. Biol. 25 (12), 4934–4945.
Kaul, R., Murakami, M., Choudhuri, T., Robertson, E.S., 2007. Epstein–Barr virus latent nuclear antigens can induce metastasis in a nude mouse model. J. Virol. 81 (19), 10352–10361.
Kawakami, H., Tomita, M., Matsuda, T., Ohta, T., Tanaka, Y., Fujii, M., Hatano, M., Tokuhisa, T., Mori, N., 2005. Transcriptional activation of survivin through the NF-kappaB pathway by human T-cell leukemia virus type I tax. Int. J. Cancer 115 (6), 967–974.
Kennedy, G., Komano, J., Sugden, B., 2003. Epstein–Barr virus provides a survival factor to Burkitt's lymphomas. Proc. Natl Acad. Sci. USA 100 (24), 14269–14274.
Knight, J.S., Cotter II, M.A., Robertson, E.S., 2001. The latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus transactivates the telomerase reverse transcriptase promoter. J. Biol. Chem. 276 (25), 22971–22978.
Kobayashi, K., Hatano, M., Otaki, M., Ogasawara, T., Tokuhisa, T., 1999. Expression of a murine homologue of the inhibitor of apoptosis protein is related to cell proliferation. Proc. Natl Acad. Sci. USA 96 (4), 1457–1462.
Lee, C.W., Raskett, C.M., Prudovsky, I., Altieri, D.C., 2008. Molecular dependence of estrogen receptor-negative breast cancer on a notch–survivin signaling axis. Cancer Res. 68 (13), 5273–5281.
Leight, E.R., Sugden, B., 2000. EBNA-1: a protein pivotal to latent infection by Epstein–Barr virus. Rev. Med. Virol. 10 (2), 83–100.
Li, F., Altieri, D.C., 1999. Transcriptional analysis of human survivin gene expression. Biochem. J. 344 (Pt 2), 305–311.
Lin, A., Wang, S., Nguyen, T., Shire, K., Frappier, L., 2008. The EBNA1 protein of Epstein–Barr virus functionally interacts with Brd4. J. Virol. 82 (24), 12009–12019.
Lu, J., Verma, S.C., Murakami, M., Cai, Q., Kumar, P., Xiao, B., Robertson, E.S., 2009. Latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus (KSHV) upregulates survivin expression in KSHV-associated B-lymphoma cells and contributes to their proliferation. J. Virol. 83 (14), 7129–7141.
Marechal, V., Dehee, A., Chikhi-Brachet, R., Piolot, T., Coppey-Moisan, M., Nicolas, J.C., 1999. Mapping EBNA-1 domains involved in binding to metaphase chromosomes. J. Virol. 73 (5), 4385–4392.
Marusawa, H., Matsuzawa, S., Welsh, K., Zou, H., Armstrong, R., Tamm, I., Reed, J.C., 2003. HBXIP functions as a cofactor of survivin in apoptosis suppression. EMBO J. 22 (11), 2729–2740.
Murakami, M., Lan, K., Subramanian, C., Robertson, E.S., 2005. Epstein–Barr virus nuclear antigen 1 interacts with Nm23-H1 in lymphoblastoid cell lines and inhibits its ability to suppress cell migration. J. Virol. 79 (3), 1559–1568.
Nayyar, V.K., Shire, K., Frappier, L., 2009. Mitotic chromosome interactions of Epstein–Barr nuclear antigen 1 (EBNA1) and human EBNA1-binding protein 2 (EBP2). J. Cell Sci. 122 (Pt 23), 4341–4350.
Nilsson, T., Zetterberg, H., Wang, Y.C., Rymo, L., 2001. Promoter-proximal regulatory elements involved in oriP-EBNA1-independent and -dependent activation of the Epstein–Barr virus C promoter in B-lymphoid cell lines. J. Virol. 75 (13), 5796–5811.
O'Neil, J.D., Owen, T.J., Wood, V.H., Date, K.L., Valentine, R., Chukwuma, M.B., Arrand, J.R., Dawson, C.W., Young, L.S., 2008. Epstein–Barr virus-encoded EBNA1 modulates the AP-1 transcription factor pathway in nasopharyngeal carcinoma cells and enhances angiogenesis in vitro. J. Gen. Virol. 89 (Pt 11), 2833–2842.
Pina-Oviedo, S., Urbanska, K., Radhakrishnan, S., Sweet, T., Reiss, K., Khalili, K., Del Valle, L., 2007. Effects of JC virus infection on anti-apoptotic protein survivin in progressive multifocal leukoencephalopathy. Am. J. Pathol. 170 (4), 1291–1304.
Raab-Traub, N., 2002. Epstein–Barr virus in the pathogenesis of NPC. Semin. Cancer Biol. 12 (6), 431–441.
Riccardi, C., Nicoletti, I., 2006. Analysis of apoptosis by propidium iodide staining and flow cytometry. Nat. Protoc. 1 (3), 1458–1461.
Rickinson, A.B., Kieff, E., 1996. Epstin–Barr virusIn: Fields, B.N., Knipe, D.M., Howley, P.M. (Eds.), Fields Virology, 3rd ed. Lippincott-Raven Publisher, Philadelphia, PA, pp. 2397–2466.
Rickinson, A.B., Kieff, E., 2001. Epstein–Barr virus, In: Knipe, D.M., Howley, P.M. (Eds.), Fields Virology, 4th ed. Lippincott/The Williams & Wilkins Co., Philadelphia, PA, pp. 2575–2627.
Saridakis, V., Sheng, Y., Sarkari, F., Holowaty, M.N., Shire, K., Nguyen, T., Zhang, R.G., Liao, J., Lee, W., Edwards, A.M., Arrowsmith, C.H., Frappier, L., 2005. Structure of the p53 binding domain of HAUSP/USP7 bound to Epstein–Barr nuclear antigen 1 implications for EBV-mediated immortalization. Mol. Cell 18 (1), 25–36.
Sarkari, F., Sanchez-Alcaraz, T., Wang, S., Holowaty, M.N., Sheng, Y., Frappier, L., 2009. EBNA1-mediated recruitment of a histone H2B deubiquitylating complex to the Epstein–Barr virus latent origin of DNA replication. PLoS Pathog. 5 (10), e1000624.
Sears, J., Ujihara, M., Wong, S., Ott, C., Middeldorp, J., Aiyar, A., 2004. The amino terminus of Epstein–Barr Virus (EBV) nuclear antigen 1 contains AT hooks that facilitate the replication and partitioning of latent EBV genomes by tethering them to cellular chromosomes. J. Virol. 78 (21), 11487–11505.
Sivachandran, N., Sarkari, F., Frappier, L., 2008. Epstein–Barr nuclear antigen 1 contributes to nasopharyngeal carcinoma through disruption of PML nuclear bodies. PLoS Pathog. 4 (10), e1000170.

Subramanian, C., Cotter II, M.A., Robertson, E.S., 2001. Epstein–Barr virus nuclear protein EBNA-3C interacts with the human metastatic suppressor Nm23-H1: a molecular link to cancer metastasis. Nat. Med. 7 (3), 350–355.

Sugden, B., Warren, N., 1989. A promoter of Epstein–Barr virus that can function during latent infection can be transactivated by EBNA-1, a viral protein required for viral DNA replication during latent infection. J. Virol. 63 (6), 2644–2649.

Sung, N.S., Wilson, J., Davenport, M., Sista, N.D., Pagano, J.S., 1994. Reciprocal regulation of the Epstein–Barr virus BamHI-F promoter by EBNA-1 and an E2F transcription factor. Mol. Cell. Biol. 14 (11), 7144–7152.

Tamm, I., Wang, Y., Sausville, E., Scudiero, D.A., Vigna, N., Oltersdorf, T., Reed, J.C., 1998. IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. Cancer Res. 58 (23), 5315–5320.

Tsimbouri, P., Drotar, M.E., Coy, J.L., Wilson, J.B., 2002. bcl-xL and RAG genes are induced and the response to IL-2 enhanced in EmuEBNA-1 transgenic mouse lymphocytes. Oncogene 21 (33), 5182–5187.

Valentine, R., Dawson, C.W., Hu, C., Shah, K.M., Owen, T.J., Date, K.L., Maia, S.P., Shao, J., Arrand, J.R., Young, L.S., O'Neil, J.D., 2010. Epstein–Barr virus-encoded EBNA1 inhibits the canonical NF-kappaB pathway in carcinoma cells by inhibiting IKK phosphorylation. Mol. Cancer 9, 1.

Verma, S.C., Robertson, E.S., 2003. ORF73 of herpesvirus Saimiri strain C488 tethers the viral genome to metaphase chromosomes and binds to cis-acting DNA sequences in the terminal repeats. J. Virol. 77 (23), 12494–12506.

Verma, S.C., Choudhuri, T., Kaul, R., Robertson, E.S., 2006. Latency-associated nuclear antigen (LANA) of Kaposi's sarcoma-associated herpesvirus interacts with origin recognition complexes at the LANA binding sequence within the terminal repeats. J. Virol. 80 (5), 2243–2256.

Verma, S.C., Choudhuri, T., Robertson, E.S., 2007. The minimal replicator element of the Kaposi's sarcoma-associated herpesvirus terminal repeat supports replication in a semiconservative and cell-cycle-dependent manner. J. Virol. 81 (7), 3402–3413.

Wilson, J.B., Bell, J.L., Levine, A.J., 1996. Expression of Epstein–Barr virus nuclear antigen-1 induces B cell neoplasia in transgenic mice. EMBO J. 15 (12), 3117–3126.

Wood, V.H., O'Neil, J.D., Wei, W., Stewart, S.E., Dawson, C.W., Young, L.S., 2007. Epstein–Barr virus-encoded EBNA1 regulates cellular gene transcription and modulates the STAT1 and TGFbeta signaling pathways. Oncogene 26 (28), 4135–4147.

Wu, H., Ceccarelli, D.F., Frappier, L., 2000. The DNA segregation mechanism of Epstein–Barr virus nuclear antigen 1. EMBO Rep. 1 (2), 140–144.

Yang, L., Cao, Z., Li, F., Post, D.E., Van Meir, E.G., Zhong, H., Wood, W.C., 2004. Tumor-specific gene expression using the survivin promoter is further increased by hypoxia. Gene Ther. 11 (15), 1215–1223.

Yates, J.L., 1988. Dissection of DNA replication and enhancer activation functions of Epstein–Barr virus nuclear antigen 1. Cancer Cell 6, 197–205.

Yates, J.L., Warren, N., Sugden, B., 1985. Stable replication of plasmids derived from Epstein–Barr virus in various mammalian cells. Nature 313 (6005), 812–815.

Yates, J.L., Camiolo, S.M., Ali, S., Ying, A., 1996. Comparison of the EBNA1 proteins of Epstein–Barr virus and herpesvirus papio in sequence and function. Virology 222 (1), 1–13.

Yin, Q., Flemington, E.K., 2006. siRNAs against the Epstein Barr virus latency replication factor, EBNA1, inhibit its function and growth of EBV-dependent tumor cells. Virology 346 (2), 385–393.

Young, L.S., Rickinson, A.B., 2004. Epstein–Barr virus: 40 years on. Nat. Rev. Cancer 4 (10), 757–768.

Zhang, X., Dong, N., Yin, L., Cai, N., Ma, H., You, J., Zhang, H., Wang, H., He, R., Ye, L., 2005. Hepatitis B virus X protein upregulates survivin expression in hepatoma tissues. J. Med. Virol. 77 (3), 374–381.

Zhou, Y., Gong, G.Z., 2006. Hepatitis C virus NS5A protein upregulates survivin gene expression. Zhonghua Gan Zang Bing Za Zhi 14 (6), 414–417.

Zhu, Y., Roshal, M., Li, F., Blackett, J., Planelles, V., 2003. Upregulation of survivin by HIV-1 Vpr. Apoptosis 8 (1), 71–79.