

Regulation of Sp100A Subnuclear Localization and Transcriptional Function by EBNA-LP and Interferon

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Epstein-Barr virus (EBV) efficiently immortalizes human B cells and is associated with several human malignancies. The EBV transcriptional activating protein EBNA2 and the EBNA2 coactivator EBNA-leader protein (EBNA-LP) are important for B cell immortalization. Recent observations from our laboratory indicate that EBNA-LP coactivation function is mediated through interactions with the interferon-inducible gene (ISG) Sp100, resulting in displacement from its normal location in promyelocytic leukemia nuclear bodies (PML NBs) into the nucleoplasm. The EBNA-LP- and interferon-mediated mechanisms that regulate Sp100 subnuclear localization and transcriptional function remain undefined. To clarify these issues, we generated a panel of Sp100 mutant proteins to ascertain whether EBNA-LP induces Sp100 displacement from PML NBs by interfering with Sp100 dimerization or through other domains. In addition, we tested EBNA-LP function in interferon-treated cells. Our results indicate that Sp100 dimerization, PML NB localization, and EBNA-LP interaction domains overlap significantly. We also show that IFN- β does not inhibit EBNA-LP coactivation function. The results suggest that EBNA-LP might play a role in EBV-evasion of IFN-mediated antiviral responses.

Introduction

EPSTEIN-BARR VIRUS (EBV), a gammaherpesvirus, is a primary cause of infectious mononucleosis and is linked with several human malignancies including, endemic Burkitt's lymphoma (eBL), nasopharyngeal carcinoma (NPC), and lymphomas in the immunosuppressed (Blaho and Aaronson 1999; Bornkamm and Hammerschmidt 2001; Thorley-Lawson 2001; Young and Rickinson 2004). EBV efficiently immortalizes human B cells (Rowe 2001; Thorley-Lawson 2001). The gene-expression program associated with B cell immortalization includes the EBV nuclear antigens (EBNAs) and latent membrane proteins (LMPs) (Thorley-Lawson 2001). Genetic and biochemical evidence has shown that EBNA2 and EBNA-leader protein (EBNA-LP) are important for initiating and maintaining EBV-mediated B cell immortalization (Hammerschmidt and Sugden 1989; Mannick and others 1991; Kempkes and others 1995; Gordadze and others 2004). EBNA2 is a transcription-activating protein, which regulates viral latency genes through mimicry of Notch signaling pathways (Fahraeus and others 1990; Tsang and others 1991; Laux and others 1994; Hsieh and others 1996; Gordadze and others 2001; Hofelmayr and others 2001) and EBNA-LP coactivates EBNA2, preferentially enhancing the expression of the major viral oncoprotein

LMP1 (Allday and others 1989; Rooney and others 1989; Harada and Kieff 1997; Nitsche and others 1997; Peng and others 2005).

EBNA-LP is an unusual protein comprising a variable number of 66 amino acid repeats and a unique 45-residue carboxyl-terminus (Sample and others 1986; Speck and others 1986) (Fig. 1). EBNA-LP plays an important role in establishment of B cell immortalization as mutant viruses lacking the Y1Y2 exons, which code for the unique carboxy-terminal domain, are much less efficient at immortalizing B cells relative to wild-type viruses (Hammerschmidt and Sugden 1989; Mannick and others 1991). Interestingly, EBNA2 coactivation function does not require the carboxy-terminal unique domain (Harada and Kieff 1997; Nitsche and others 1997). The EBNA2 coactivation function of EBNA-LP requires a minimum of two repeats, and deletion of evolutionarily conserved region 3 (CR3) in EBNA-LP greatly diminishes its coactivation function (Nitsche and others 1997; Peng and others 2000). Recent evidence has suggested that the promyelocytic leukemia nuclear body (PML NB)-associated protein Sp100 is an important mediator of EBNA-LP coactivation function (Ling and others 2005). EBNA-LP specifically relocalizes Sp100 from PML NBs, and this activity correlates positively with EBNA2 coactivation.

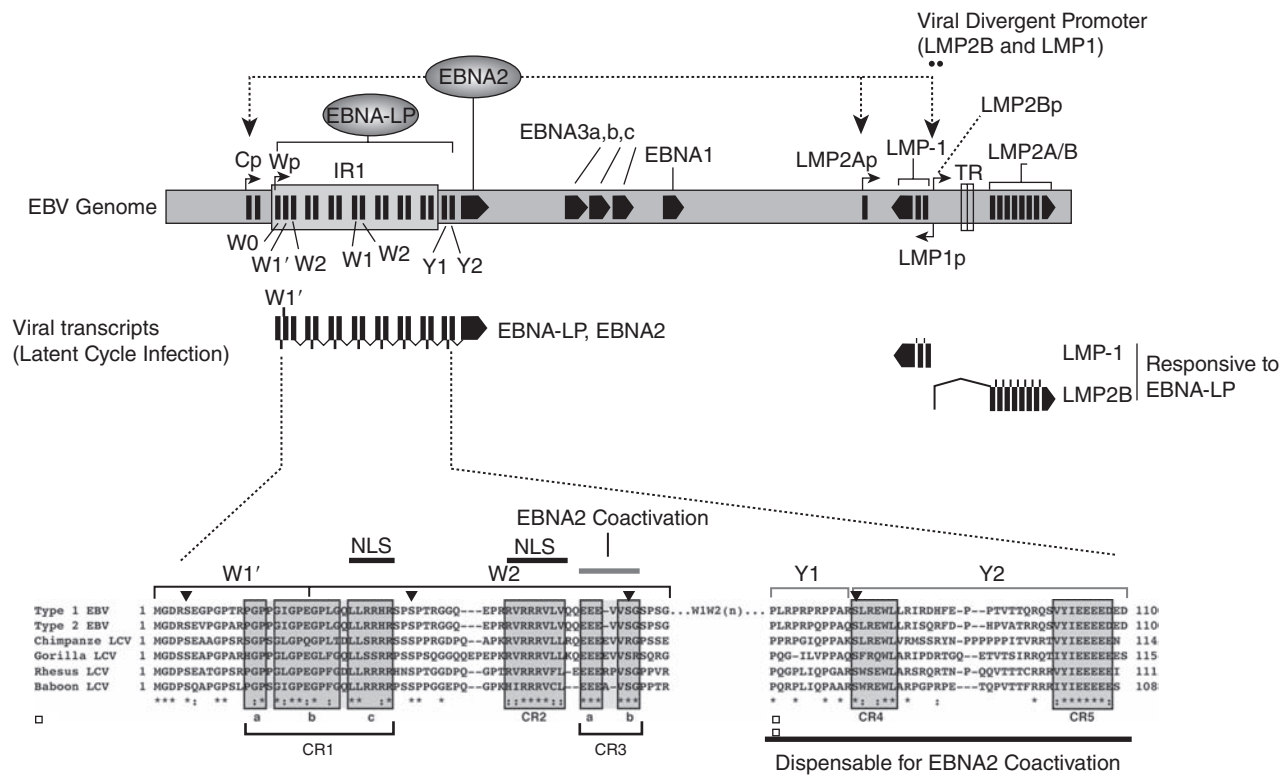


FIG. 1. Schematic of the Epstein-Barr Virus (EBV) genome and the sequence of EBNA-LP. The EBV genome is shown in linear form with relevant structural motifs and location of exons (black boxes) encoding latency proteins, which are labeled above. The internal repeat region (IR1) is also shown by the gray box. The viral transcript encoding EBNA-LP is shown below and is derived from the latency W promoter (Wp). The first exon transcribed from Wp is W0 and is noncoding. Alternative splicing from W0 to W1 can result in formation of an initiation codon (W1') or no initiation codon (W1). Splicing that generates W1' gives rise to transcripts encoding EBNA-LP. EBNA-LP is thus composed of repeated W1 and W2 exons as well as two unique exons known as Y1 and Y2. The transcript is bi-cistronic as it also contains the EBNA2 ORF at the 3' end. The dashed line from the EBNA2 protein indicates promoters that are activated by EBNA2. The viral bidirectional promoter, which controls expression of LMP-1 and LMP2B, is indicated by asterisks. Transcripts encoding LMP-1 are shown below. A sequence comparison between EBV EBNA-LP and other nonhuman primate lymphocryptoviruses is shown below indicating presence of conserved regions (CR). Location of nuclear localization signals (NLS) and regions required for EBNA2 coactivation are indicated. Inverted triangles above the sequence indicate conserved serine residues.

Promyelocytic leukemia nuclear bodies have been shown to regulate a broad range of cellular processes including apoptosis, genomic stability, cell cycle regulation, transcriptional regulation, and antiviral responses (Everett and others 1998; Muller and Dejean 1999; Seeler and Dejean 1999; Adamson and Kenney 2001; Borden 2002; Bernardi and Pandolfi 2003; Ling and others 2005; Bernardi and Pandolfi 2007; Everett and Chelbi-Alix 2007). Proteins endogenously expressed in PML NBs include Sp100, PML, Daxx, and small ubiquitin-related modifiers such as SUMO (Negorev and Maul 2001). The PML protein is a definitive marker for PML NBs and is required for their proper assembly (Ishov and others 1999). Other proteins conditionally expressed in or in close proximity to PML NBs, include heterochromatin protein 1 α (HP-1 α), CREB-binding protein (CBP), and the tumor suppressors p53 and pRb (Negorev and Maul 2001).

Several herpesviruses have been shown to interact with or degrade PML and PML NB-associated proteins, suggesting

that these nuclear organelles are important modulators of virus infection. For example, expression of the immediate early gene product ICP0 by herpes simplex virus-1 (HSV-1) has been shown to induce the proteasome-dependent degradation of PML (Everett and others 1998; Chelbi-Alix and de The 1999). This results in the transient association of PML NBs that are first found associated with incoming genomes (Everett and others 2003). HSV-1 viruses expressing ICP0 mutants unable to induce PML degradation have a much lower probability of progressing into productive lytic infection at low MOIs (Everett and others 2006). In addition, extensive depletion of PML in human fibroblast cells significantly increases gene expression and plaque formation of ICP0 null viruses (Everett and others 2006). Moreover, viruses with mutations that inactivate ICP0 do not enter productive replication after low multiplicity infection of human fibroblasts, but instead are retained in a repressed quiescent state that in some respects resembles latency. The

CMV immediate early protein IE1 appears to induce the loss of sumoylated forms of PML, resulting in the destruction of PML NBs (Korioth and others 1996; Wilkinson and others 1998; Muller and Dejean 1999; Ahn and Hayward 2000; Lee and others 2004). Like HSV-1, CMV mutants impaired for inducing PML NB destruction are less efficient at establishing productive infection at low MOIs and these mutants can be complemented in human fibroblast cells depleted for PML protein (Mocarski and others 1996; Greaves and Mocarski 1998; Ahn and Hayward 2000; Lee and others 2004; Tavalai and others 2006). CMV also encodes a tegument protein known as pp71 that induces the degradation of the PML NB-associated protein hDaxx (Hwang and Kalejta 2007). Depletion of hDaxx enhances hCMV gene expression, especially by pp71-deficient viruses (Ishov and others 2002; Cantrell and Bresnahan 2005; Cantrell and Bresnahan 2006; Saffert and Kalejta 2007). Collectively, the data suggest that PML NB-associated proteins have a role in inhibiting virus gene expression during productive lytic infection. In addition, some of these studies suggest that PML NBs may play a role in establishment or maintenance of latency.

Both Sp100 and PML promoters contain interferon stimulated response elements and are up-regulated at the transcriptional level by interferon (Guldner and others 1992; ChelbiAlix and others 1995; Grotzinger and others 1996; Negorev and others 2006). HSV-1 replicates more efficiently in interferon-treated PML-negative fibroblasts relative to interferon-treated wild-type fibroblasts (Chee and others 2003). Interferon was also able to inhibit immediate early viral gene expression and PML degradation in human cells (Taylor and others 2000). Together, these data suggest that PML carries out interferon-mediated antiviral activities. Whether Interferon modifies the function of Sp100, however, remains undefined.

Sp100A contains several functional domains including a carboxyl-terminal nuclear localization signal (NLS), a heterochromatin-1 α interaction site, an amino-terminal dimerization domain, and a PML NB-targeting domain (Seeler and others 1998; Sternsdorf and others 1999; Negorev and others 2001; Ling and others 2005). Three additional alternatively spliced isoforms of Sp100A are made in lower abundance relative to Sp100A and localize predominantly outside of PML NBs in the nucleoplasm (Sp100-B, -C, -HMG) (Negorev and others 2006). EBNA-LP coactivation function has been linked predominantly with the PML NB-associated Sp100A isoform (Ling and others 2005). The Sp100 dimerization, PML-targeting, and EBNA-LP interacting domains reside within the amino-terminal 150 amino acid residues, but their relationship to each other remains unclear (Sternsdorf and others 1999; Negorev and others 2001; Ling and others 2005). Previous attempts to separate the PML NB localization and dimerization domains were unsuccessful. These studies utilized truncated Sp100 fusion proteins, which might not have adopted physiological conformations required for these domains to function correctly (Sternsdorf and others 1999). However, another study suggested that the Sp100 dimerization and PML NB-localization domains are distinct because fusion of Sp100 residues 29–152 to the p53-binding domain of hMDM2 resulted in loss of self-aggregation, but retention of PML-NB localization (Negorev and

others 2001). To clarify this issue, we generated a series of small deletions within residues 3–152 in the context of the full length Sp100 protein. The mutant Sp100 proteins were also used to determine the EBNA-LP binding domain relative to the dimerization and PML NB localization domains. In addition, we tested whether Interferon could modulate EBNA-LP-mediated relocalization of Sp100 from PML NBs and whether this might have an effect on EBNA-LP coactivation function.

Materials and Methods

Plasmids

A flag epitope-tagged Sp100A protein expressing the amino terminal 182 residues of Sp100 fused with the SV40 NLS was generated by PCR (1-182-Flag). Fifteen amino acid deletions were introduced into full length, hemagglutinin-tagged Sp100A by PCR as described previously (Gordadze and others 2004; Ling and others 2005). EBNA2, wild-type EBNA-LP, and Δ CR3 EBNA-LP expression plasmids, and the multimerized Cp promoter luciferase reporter plasmid (8xCp-LUC) have been described previously (Harada and Kieff 1997; Fuentes-Panana and Ling 1998). The ICP0 expression plasmid (pGH92) was a gift from Dr. Gary Hayward (Johns Hopkins University School of Medicine, Baltimore, MD, USA). The ICP0-luc plasmid was kindly provided by Dr. Gerd Maul (The Wistar Institute, Philadelphia, PA, USA).

Cell culture, interferon treatment, and transfections

EBV-positive Daudi and EBV-negative DG75 cells were grown in RPMI 1640 media supplemented with 10% fetal bovine serum (Hyclone) and maintained at 37°C in 5% CO₂. The BL41-LP cell line (a gift from Dr. Elliott Kieff, Harvard Medical School, Boston, MA, USA) was maintained in tetracycline-free media. EBNA-LP expression was induced in BL41-LP cells by adding 2 ng/mL doxycycline (Clontech). Recombinant human interferon beta (Chemicon, IF014) was added directly to the cells where indicated, at a final concentration of 100 U/mL. All transfections were carried out by electroporation (BioRad Gene Pulser) as described previously (Gordadze and others 2004). In brief, 1×10^7 cells plus 30 μ g total plasmid DNA were resuspended in 0.5 mL serum-free RPMI. Cells were pulsed at 200V and 975F, placed in 2.5 mL RPMI media plus 10% FBS, and maintained for 48 h at 37°C in 5% CO₂.

Luciferase reporter assays

Two days posttransfection, cells were solubilized and luciferase activity measured using the dual-luciferase reporter assay system (Promega) according to the manufacturer's recommendations. The remainder of the cell lysates were used to determine protein expression levels by immunoblot assays.

Coimmunoprecipitation and immunoblot assays

For coimmunoprecipitation assays, cells were harvested in 1% NP40 (Calbiochem) containing protease inhibitor

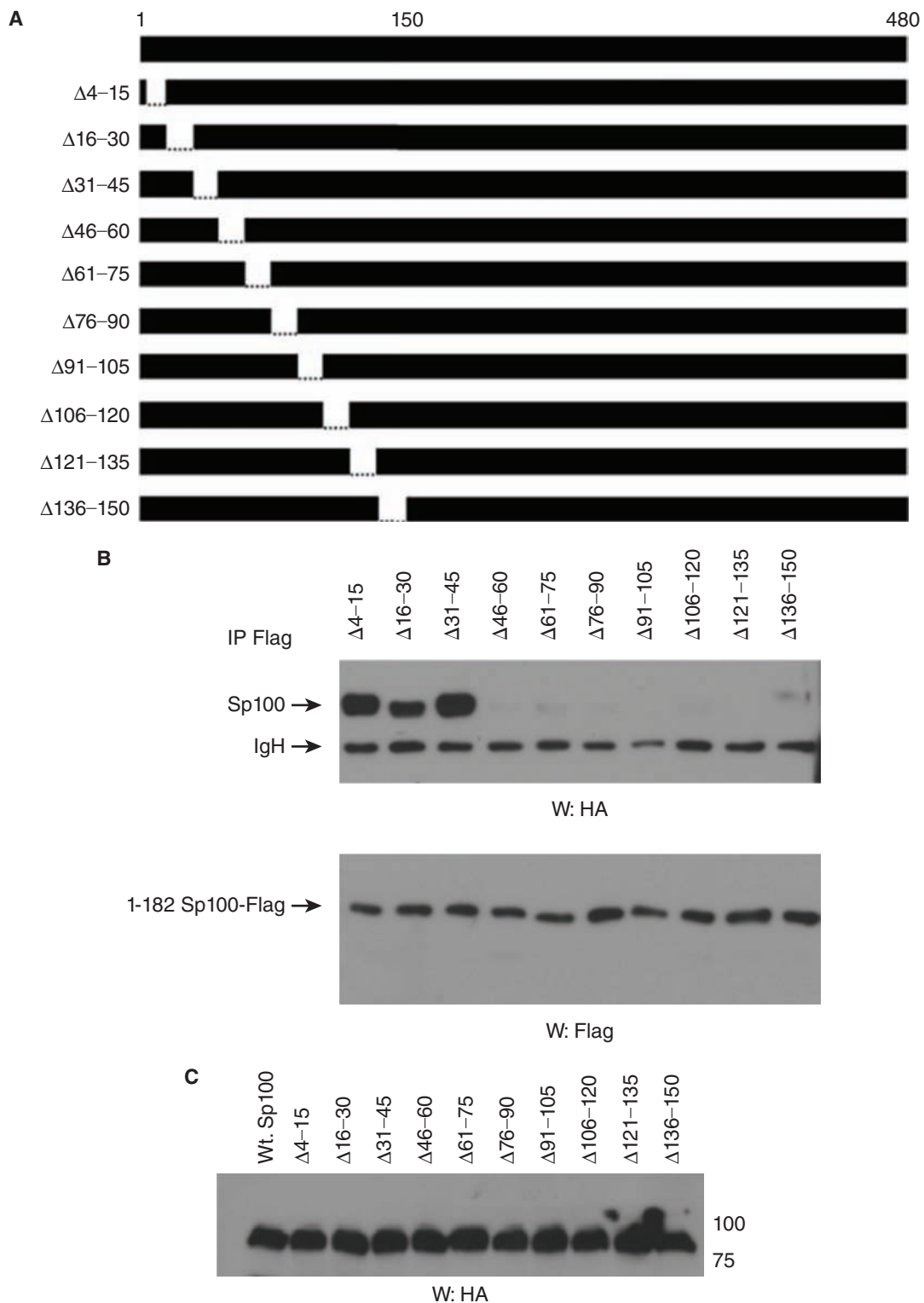


FIG. 2. Sp100A dimerization and EBNA-LP interaction domains are between amino acid residues 46–150. **(A)** Schematic representation of wild-type and mutant Sp100A. Boundaries of each deletion are indicated. **(B)** EBV-negative DG75 cells were cotransfected with 1–182 Sp100-Flag and wild-type or mutant Sp100A plasmids represented in **(A)**. Following immunoprecipitation with anti-Flag antibodies, coprecipitated Sp100A was detected by Western blot using anti-HA antibodies. Anti-Flag antibodies were used to confirm 1–182-Flag precipitation. **(C)** Western blot detection of wild-type and mutant Sp100A proteins expressed in transfected DG75 cells. **(D)** DG75 cells were cotransfected with wild-type or mutant Sp100A and flag-tagged EBNA-LP expression plasmids. Immunoprecipitations and Western blots were carried out as outlined above, using anti-Flag and anti-HA antibodies.

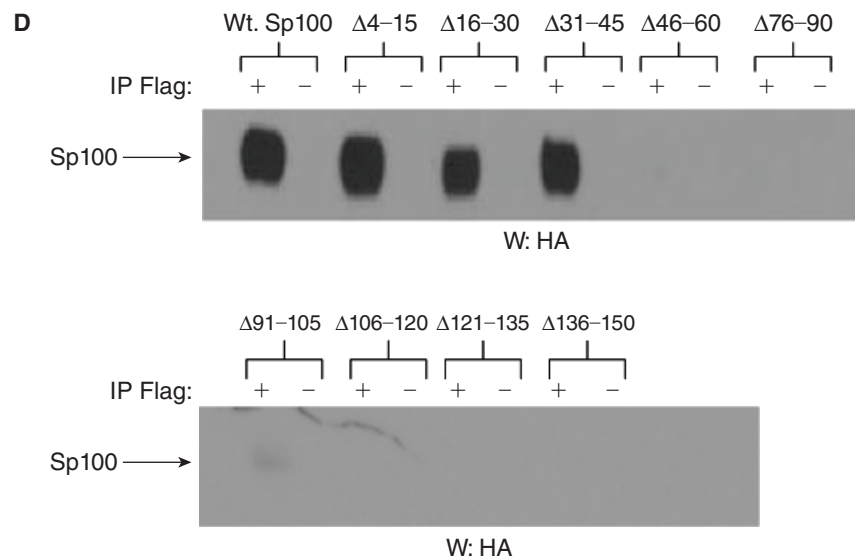


FIG. 2. Continued.

cocktail tablets (Roche). Soluble lysates were immunoprecipitated with anti-Flag antibodies at 4°C overnight. Immune complexes were pulled down with Immobilized Protein G beads (Pierce) for 1 h at room temperature. Protein complexes were recovered by boiling for 5 min in Laemmli buffer (4% SDS, 20% glycerol, 0.125 M Tris-Cl, pH 6.8, 0.004% bromophenol blue, 3% 2-mercaptoethanol). For immunoblot analyses, lysates were resolved on a 7.5% SDS-PAGE gel, immobilized onto nitrocellulose membranes, and blocked in 5% milk in phosphate-buffered saline (PBS). EBNA2 and EBNA-LP protein expression were detected using rat monoclonal anti-EBNA2 antibodies or mouse monoclonal anti-EBNA-LP antibodies respectively (Finke and others 1987; Kremmer and others 1995). Transfected Sp100A proteins were detected using anti-HA (Covance) or Flag antibodies (M2, Sigma). Endogenous Sp100 protein expression was determined by probing the blots with rabbit polyclonal anti-Sp100 antibodies, which were generated by immunizing rabbits with a His-tagged Sp100 protein comprised of the amino-terminal 252 residues purified from *Escherichia coli*. PML protein expression was monitored using rabbit polyclonal anti-PML (H-238, Santa Cruz), while α -tubulin expression was determined using mouse monoclonal anti- α -tubulin antibodies (Sigma). All primary antibody incubations were carried out in 0.5% milk in PBS, while secondary antibody reactions were carried out using antirat (EBNA2), antimouse (HA, Flag, EBNA-LP, α -tubulin) or antirabbit (Sp100) HRP-conjugated antibodies (Jackson Immunolaboratories) in a solution of 0.01% Tween-20/PBS. Blots were developed using a chemiluminescence detection kit (Pierce).

Immunofluorescence and co-localization

Transfected or IFN-treated cells were spun onto coverslips at 1,000 rpm for 1 min. The cells were fixed in 4% formaldehyde, and permeabilized in 0.5% Triton X-100 (Fisher) (Ling and others 2005). Permeabilized cells were

incubated at 4°C overnight in 5% milk/TBST/0.02% Sodium azide (blocking buffer). Mouse monoclonal anti-HA antibodies (Covance), followed by Alexa Fluor 594 goat antimouse IgG (Molecular probes) secondary antibodies were used to detect localization of HA-tagged Sp100A proteins. Endogenous Sp100 expression and localization was monitored using rabbit -polyclonal anti-Sp100 antibodies (Calbiochem) followed by Alexa Fluor 488 goat antirabbit IgG secondary antibodies (Molecular Probes). PML localization was detected using primary anti-PML monoclonal or polyclonal antibodies (Santa Cruz) followed by Alexa Fluor 594 goat antimouse or antirabbit secondary antibodies. ICP0 expression and localization was detected using mouse monoclonal antibodies (Eastcoast Bio). The cells were visualized and counted using a Zeiss AxioPlan fluorescence microscope (Integrated Microscopy Core, BCM). To determine the numbers of cells displaying punctate or diffuse Sp100 expression, we counted 100 cells each, in triplicate experiments. The numbers of Sp100-associated dots were also determined as a function of time and IFN- β dose in BL41-LP cells.

Results

Sp100 dimerization and EBNA-LP-interaction domains reside between amino acid residues 46-150

Biochemical characterization of Sp100A has revealed that the amino-terminal 152 residues mediate its dimerization, interaction with EBNA-LP, and localization to PML NBs (Sternsdorf and others 1999; Negorev and others 2001; Ling and others 2005). To further define the Sp100A residues most important for these functions, we generated a panel of Sp100A proteins with 15 amino acid deletions within the amino-terminal 150 amino acid residues (Fig. 2A). Dimerization was assessed by analyzing the ability of a coexpressed truncated Sp100 protein (1-182-Flag) to coimmunoprecipitate wild-

type or mutant Sp100A proteins as described previously (Sternsdorf and others 1999). Deletions spanning amino acid residues 46–150 were deficient for Sp100A self-association, while all other deletions tested did not affect Sp100 self-association (Fig. 2B). Control blots indicated that all of the proteins were expressed at similar levels (Figs. 2B and C). To determine the specific EBNA-LP binding domain, EBNA-LP-Flag was coexpressed with the Sp100 mutant proteins and their association with each other was analyzed by coimmunoprecipitation. EBNA-LP was able to co-immunoprecipitate Sp100 with deletions in residues 4–45, but failed to associate with Sp100A mutants with deletions spanning residues 46–150 (Fig. 2D). Collectively, these data suggest that Sp100A dimerization and EBNA-LP-binding domains localize between amino acid residues 46–150.

The Sp100A PML NB targeting domain maps to residues 46–150

Immunofluorescence assays were carried out to determine the cellular localization of the Sp100A deletion mutants described in Figure 2A. Wild-type Sp100A was localized in punctate nuclear dots characteristic of PML NBs (Fig. 3). Amino acid deletions within Sp100A residues 4–45 showed similar staining patterns as wild-type Sp100A while Sp100A mutants with deletions spanning residues 46–150 showed diffuse nuclear localization (Fig. 3, selected mutants shown to illustrate general pattern). The percentage of cells expressing wild-type or mutant Sp100 proteins as punctate nuclear dots or diffuse nuclear staining, is shown in Table 1. Co-staining with a PML antibody confirmed that

those proteins with a punctate staining pattern were within PML NBs (data not shown). These data suggest that the amino terminal 45 residues of Sp100A are not important for Sp100A localization to PML nuclear bodies, and PML NB targeting function is mediated by a domain(s) contained between amino acid residues 46–150.

EBNA-LP function is not inhibited by interferon

Type I and II IFNs induce Sp100 and PML expression, resulting in an increase in the number and size of PML NBs (Guldner and others 1992; ChelbiAlix and others 1995; Grotzinger and others 1996). Previous studies have shown that PML contributes to IFN-induced effects that inhibit HSV-1 replication (Chee and others 2003; Everett and others 2006). HSV-1 ICP0 and EBV EBNA-LP functions have been linked to their association with, and modulation of PML NBs (Ling and others 2005; Everett and others 2006). We therefore questioned whether IFN-induced Sp100A accumulation in PML NBs might inhibit EBNA-LP function. The IFN-inducibility of Sp100 was first confirmed in EBV-positive Daudi and BL41-LP cell lines (Fig. 4A). We also showed that the numbers of Sp100-associated nuclear dots increased as a function of IFN- β dose (Table 2) and treatment time (Table 3). As expected, IFN- β also induced PML (Figs. 4B and 5F). In addition, ICP0 induced PML degradation in untreated cells but not in those treated with IFN- β (Figs. 5I and L). Finally, IFN- β also inhibited the stimulation of the ICP0 promoter in transient transfection assays in agreement with previous reports (Negorev and others 2006) (Fig. 4C). To

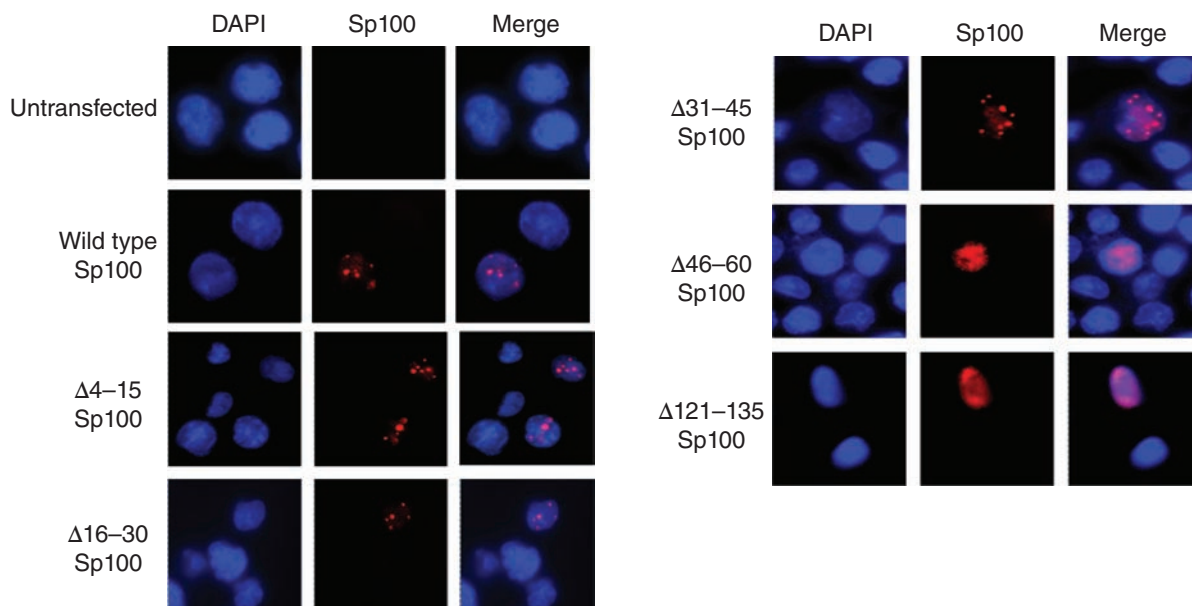


FIG. 3. The Sp100A PML NB targeting domain is between amino acid residues 46–150. Immunofluorescence detection of transiently expressed Sp100A proteins in DG75 cells. Sp100A (red) was monitored using mouse monoclonal anti-HA antibodies. Cells were co-stained with DAPI to detect the nucleus. Merged panels are shown in the far right hand column as indicated.

TABLE 1. WILD-TYPE AND MUTANT Sp100 EXPRESSION PATTERNS IN EBV-NEGATIVE DG 75 CELLS

Sp100	Expression patterns (n = 300)	
	Punctate (%)	Diffuse (%)
Wild-type	96	4
Δ4–15	98	2
Δ16–30	96	4
Δ31–45	97	3
Δ46–60	0	100
Δ121–135	0	100

determine the effects of IFN- β on EBNA-LP-induced Sp100A relocalization from PML NBs, we analyzed Sp100A by immunofluorescence in Daudi cells following IFN- β treatment and transient expression of EBNA-LP. Transient expression of EBNA-LP in Daudi cells showed characteristic diffuse nuclear staining for EBNA-LP in both untreated and IFN-treated cells (Figs. 6B and E). Surprisingly, EBNA-LP displaced Sp100 from PML NBs in IFN-treated cells (Fig. 6F).

To alleviate concerns that this observation was due to EBNA-LP overexpression, we analyzed Sp100A localization in an EBNA-LP inducible cell line BL41-LP. EBNA-LP induction resulted in the displacement of Sp100A from PML NBs in both untreated and IFN- β -treated cells (Figs. 7F and L). These results suggest that EBNA-LP's ability to relocalize Sp100 may be refractory to the effects of IFN- β on Sp100A accumulation in nuclear bodies.

To determine the effects of IFN- β on EBNA-LP coactivation function, we transfected untreated or IFN- β -treated Daudi cells with an EBNA2-responsive luciferase reporter. In untreated and IFN- β -treated cells, EBNA-LP coactivated EBNA2 by about 16-fold (Figs. 8A and B). No coactivation was observed when EBNA2 was coexpressed with a previously characterized coactivation deficient EBNA-LP mutant (Δ CR3LP).

Discussion

In this study, we found that the Sp100A EBNA-LP binding domain lies between residues 46–150, which overlap with its dimerization and PML NB localization domains. Furthermore, under conditions in which IFN- β inhibited

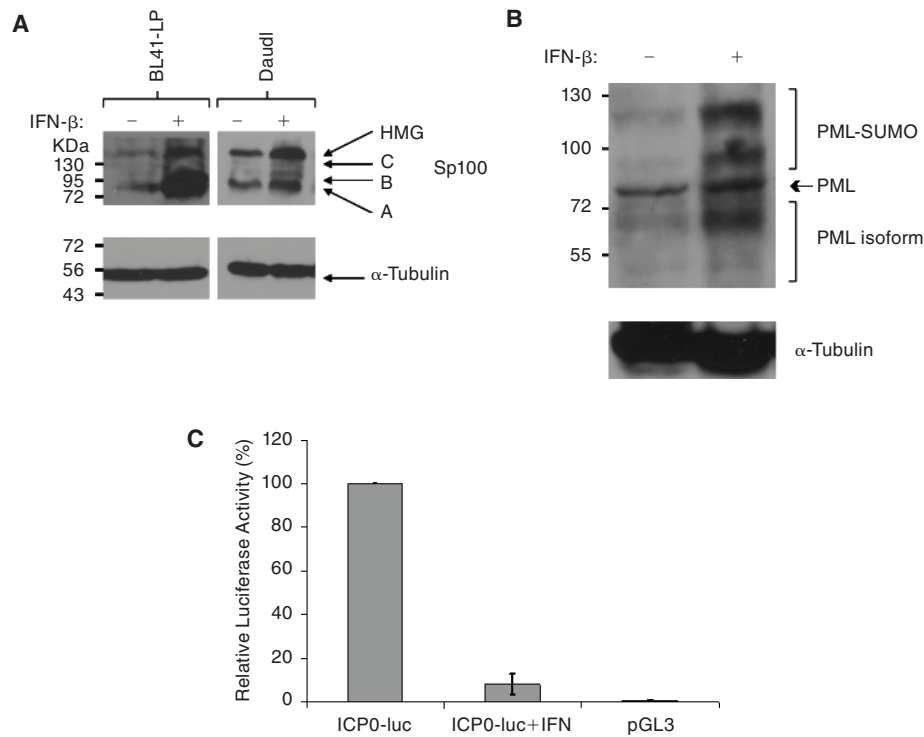


FIG. 4. IFN- β regulates Sp100 and PML expression and the ICP0 promoter. (A) Western blot of endogenous Sp100A expression in BL41-LP and Daudi cells treated (+) or untreated (-) with 100 U/mL IFN- β for 24 h. Molecular weight markers are shown to the left side of the blot and Sp100 isoforms are indicated on the right side. α -Tubulin controls are shown in the panel below. (B) Western blot of endogenous PML expression in Daudi cells treated (+) or untreated (-) with 100 U/mL IFN- β for 24 h. Molecular weight markers are shown to the left side of the blot and PML isoforms indicated on the right side. α -Tubulin controls are shown in the panel below. (C) ICP0 promoter activity is inhibited by IFN- β . ICP0 promoter-driven luciferase plasmids were transfected into Daudi cells treated (+) and untreated (-) with IFN- β . A control sample was transfected with empty vector (pGL3). Relative luciferase activity for each of the transfections is indicated on the y-axis. Standard errors are indicated.

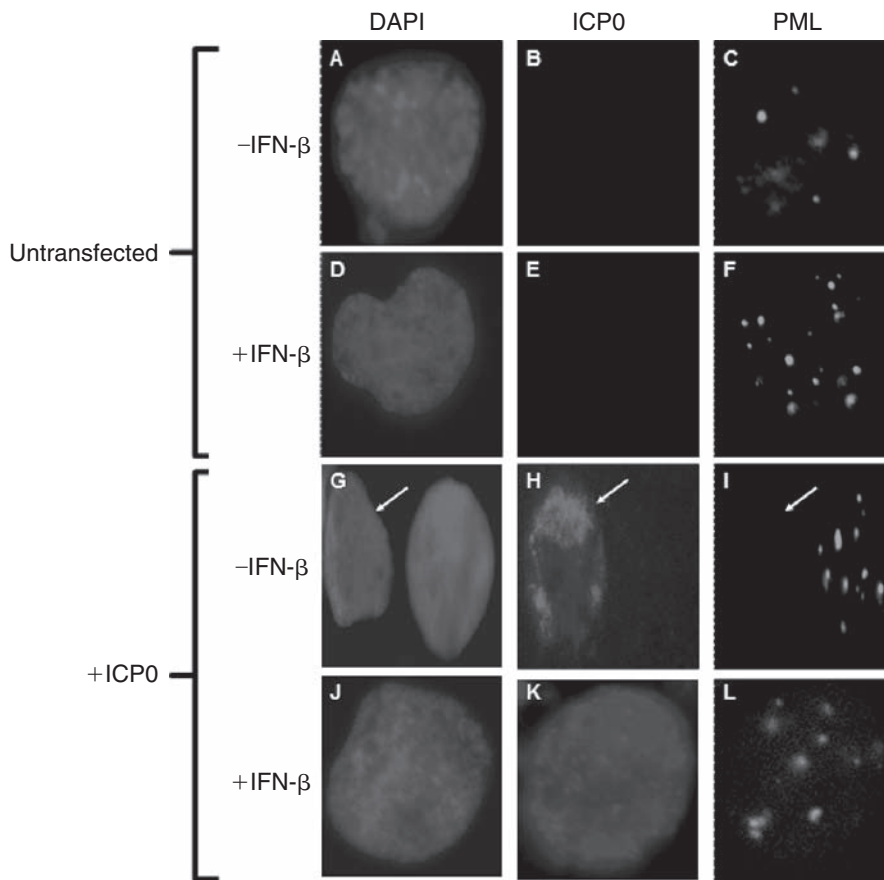


FIG. 5. HSV-1 ICP0-mediated PML degradation is inhibited by IFN- β . Immunofluorescence detection of ICP0 and Sp100A in Daudi cells. Untransfected Daudi cells without IFN (-) or treated with IFN (+) for 24 h (+) are indicated on the left side of the panels. Daudi cells transfected with an ICP0 expression plasmid without IFN (-) or treated with IFN (+) for 24 h (+) are indicated similarly.

HSV-1 ICP0-induced PML degradation and ICP0 promoter activity, EBNA-LP coactivation function was unimpaired. The results implicate a previously uncharacterized role for EBNA-LP in resistance against host innate immune responses.

The results we obtained from characterizing Sp100A dimerization, EBNA-LP binding, and PML NB localization

domains are consistent with previous findings (Sternsdorf and others 1999; Negorev and others 2001; Ling and others 2005). We were able to extend these studies by using more refined mutations in the context of the full length Sp100 protein. Although we were unable to separate domains for each of these functions, our data do not preclude the possibility that they are distinct. One explanation for our findings is

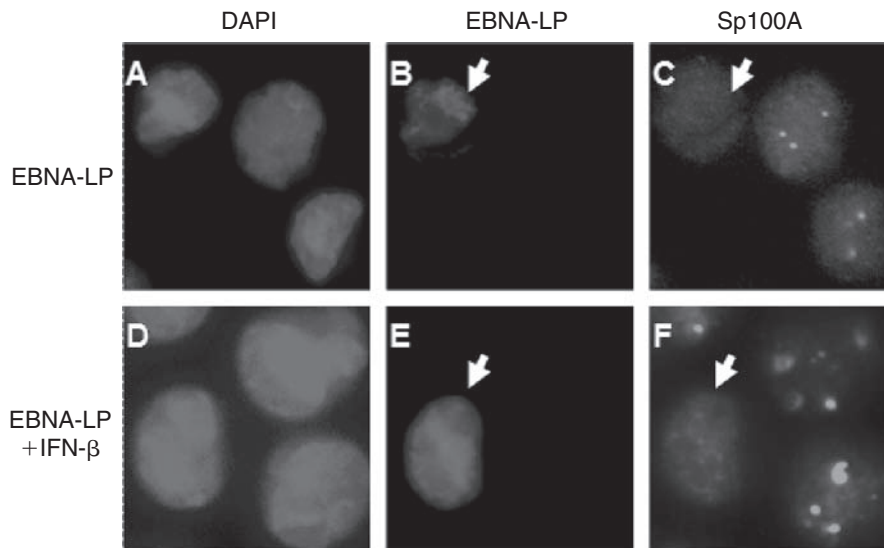


FIG. 6. EBNA-LP displaces Sp100 from PML NBs in IFN-treated cells. Immunofluorescence of Daudi cells transfected with an EBNA-LP expression plasmid with or without IFN treatment. EBNA-LP and Sp100A were detected using monoclonal and polyclonal antibodies respectively as described in the Materials and Methods. DAPI staining of each field is shown in the panels on the left.

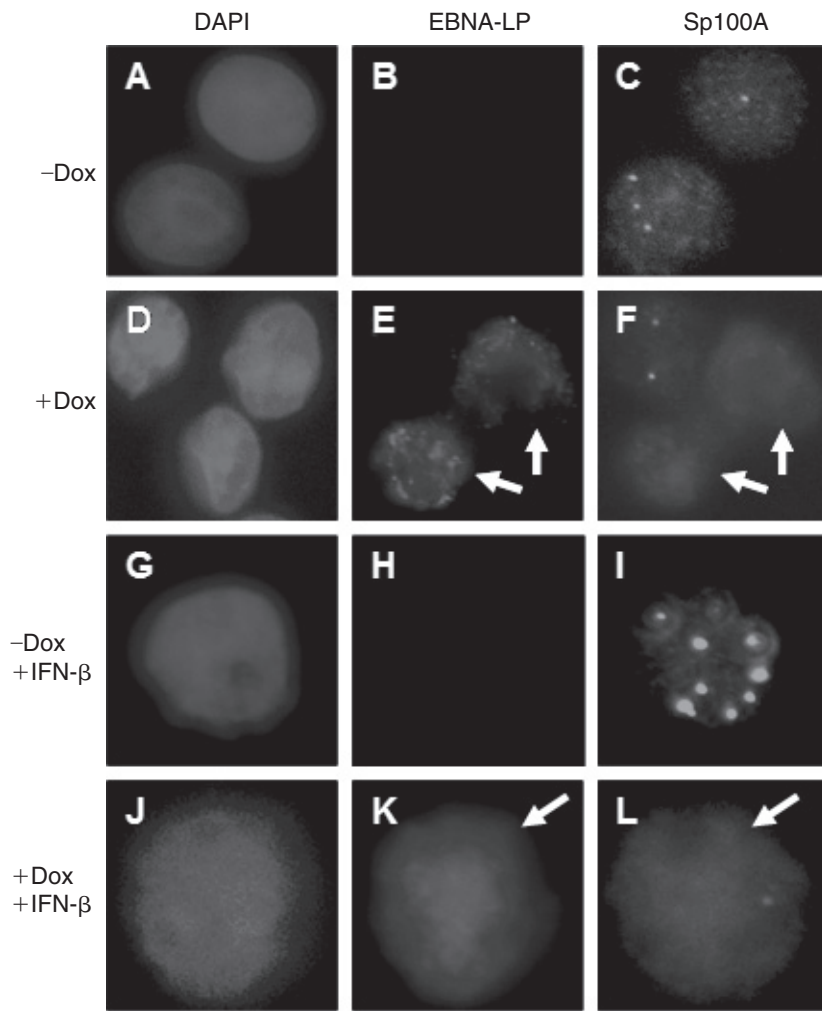


FIG. 7. Induction of EBNA-LP expression in BL41-LP cells displaces Sp100A. BL41-LP cells were treated with 2 ng/mL doxycycline (Dox), resulting in the induction of EBNA-LP expression in about 50–80% of the cells. EBNA-LP and Sp100A were detected with monoclonal anti-EBNA-LP and polyclonal Sp100 antibodies respectively. Addition of Dox and/or IFN- β is shown on the left of each series of panels. White arrows indicate cells expressing EBNA-LP.

that the Sp100A amino acid residues 46–150 may form interdependent tertiary structures, such that mutations in one region affect the function of adjacent regions. Therefore, more targeted subtle mutations may need to be introduced within residues 46–150 in order to functionally distinguish these domains. Alternatively, the Sp100A dimerization domain could mediate heterodimerization with EBNA-LP and/or cellular factors that modulate Sp100A localization

within PML NBs (i.e., there is in fact only a single domain that mediates all three functions).

The ability of EBNA-LP to overcome the cellular response induced by IFN- β may play an important role during the establishment of latent infection in B cells. Several previous studies have shown that preincubation or simultaneous treatment with IFN relative to EBV exposure inhibits EBV-induced immortalization (Adams and others 1975b; Aman

TABLE 2. EFFECT OF IFN- β DOSE ON Sp100 ACCUMULATION IN BL41-LP CELLS

IFN- β dose (U/mL)	Percentage of Sp100-associated nuclear dots per cell (n = 300)		
	≤ 2	$\geq 3 \leq 4$	≥ 5
Untreated	88	9	3
10	84	14	2
50	69	19	12
100	17	22	61
500	8	19	73
1000	7	27	66

TABLE 3. TIME-DEPENDENT RESPONSE OF Sp100 TO IFN- β TREATMENT IN BL41-LP CELLS

Time after IFN- β treatment (h)	Percentage of Sp100-associated nuclear dots per cell (n = 300)		
	≤ 2	$\geq 3 \leq 4$	≥ 5
0	85	9	6
4	76	15	9
8	62	22	16
16	49	19	32
24	20	18	62

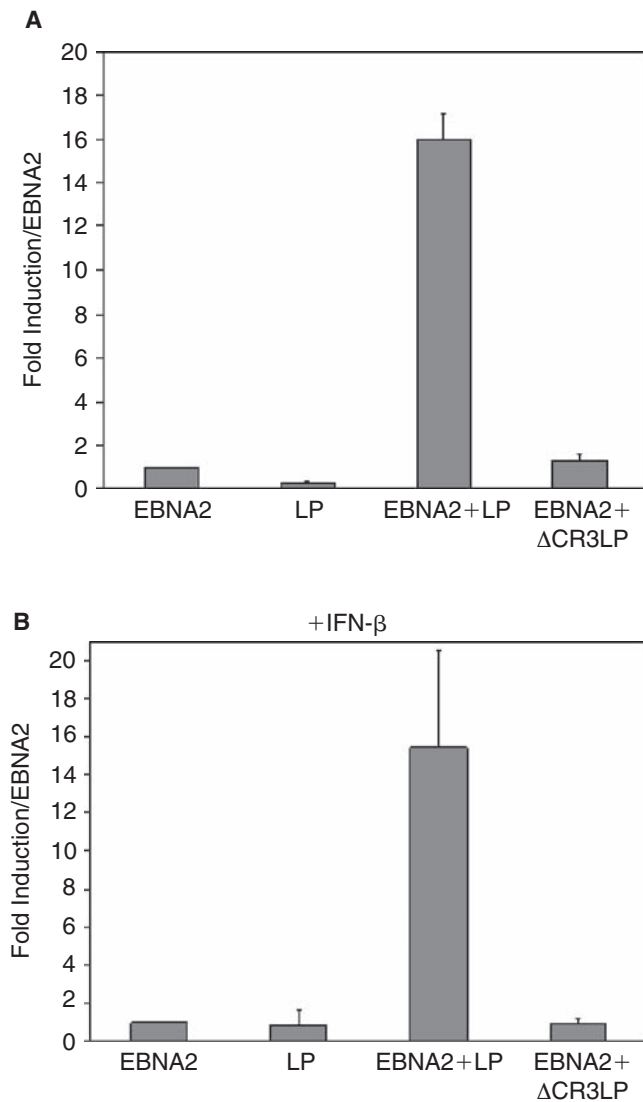


FIG. 8. EBNA-LP coactivation function is not inhibited by IFN- β . Cp promoter luciferase reporter plasmids were transfected with EBNA2, EBNA-LP (LP), EBNA2 + LP, or EBNA2 + Δ CR3LP expression plasmids. Transfected cells were either untreated (**A**) or pulsed and maintained in 100 U/mL IFN- β for 24 h posttransfection (**B**). Relative fold-induction is shown on the left of each graph. Standard errors of the mean are indicated for each bar.

and Vongabain 1990; Kanda and others 1992). However, other studies have found that addition of IFN 24–48 h following exposure to EBV had little to no effect on EBV-induced immortalization (Adams and others 1975a; Menezes and others 1976; Lotz and others 1985). Furthermore, EBV-transformed lymphoblasts are refractory to IFN-induced growth inhibition.

Several EBV proteins or gene products have been implicated in counteracting IFN-induced cellular responses, including LMP-1, EBNA2, and the small noncoding EBER

RNAs (Aman and Vongabain 1990; Zhang and Pagano 2000; Nanbo and others 2002). Our work suggests that EBNA-LP function is also refractory to IFN, which may contribute to the IFN-insensitive phenotype of EBV immortalized B cells. Although interferons are able to induce antiproliferative effects on target cells, including some malignant cell types, the potential widespread application of IFNs in controlling aberrant growth of malignant cells is hindered by IFN resistance (Adams and others 1975b; Strander 1986). Identifying viral or cellular proteins that contribute to IFN resistance will provide more insight into the mechanisms that counteract the antiproliferative actions of IFNs. Determining more definitive mechanisms of EBNA-LP action will reveal specific factors that help mediate EBNA-LP's coactivation function as well as its involvement in IFN resistance. In addition, since the gene expression pattern seen in lymphoblastoid cell lines (LCLs) mimics *in vivo* cases of EBV-associated post-transplant lymphoproliferative diseases (Straus and others 1993), our results may have some therapeutic applications. Identification of factors which mediate IFN resistance may be important in designing therapeutic agents against EBV-associated malignancies.

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