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Mediation of Epstein–Barr virus EBNA-LP transcriptional coactivation by Sp100

Paul D Ling[1,*], Rong Sheng Peng[1], Ayako Nakajima[2], Jiang H Yu[2], Jie Tan[1], Stephanie M Moses[1], Wei-Hong Yang[2], Bo Zhao[3], Elliott Kieff[3], Kenneth D Bloch[4] and Donald B Bloch[2,*]

[1]Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, TX, USA, [2]Department of Medicine, Harvard Medical School and Center for Immunology and Inflammatory Diseases of the General Medical Services, Massachusetts General Hospital, Boston, MA, USA, [3]Departments of Medicine and Microbiology and Molecular Genetics, Channing Laboratory, Brigham and Women's Hospital and Harvard Medical School, Boston, MA, USA and [4]Department of Medicine, Harvard Medical School and Cardiovascular Research Center of the General Medical Services, Massachusetts General Hospital, Boston, MA, USA

The Epstein–Barr virus (EBV) EBNA-LP protein is important for EBV-mediated B-cell immortalization and is a potent gene-specific coactivator of the viral transcriptional activator, EBNA2. The mechanism(s) by which EBNA-LP functions as a coactivator remains an important question in the biology of EBV-induced B-cell immortalization. In this study, we found that EBNA-LP interacts with the promyelocytic leukemia nuclear body (PML NB)-associated protein Sp100 and displaces Sp100 and heterochromatin protein 1α (HP1α) from PML NBs. Interaction between EBNA-LP and Sp100 was mediated through conserved region 3 in EBNA-LP and the PML NB targeting domain in Sp100. Overexpression of Sp100 lacking the N-terminal PML NB targeting domain, but not a mutant form of Sp100 lacking the HP1α interaction domain, was sufficient to coactivate EBNA2 in a gene-specific manner independent of EBNA-LP. These findings suggest that Sp100 is a major mediator of EBNA-LP coactivation. These studies indicate that modulation of PML NB-associated proteins may be important for establishment of latent viral infections, and also identify a convenient model system to investigate the functions of Sp100.

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*Corresponding authors. PD Ling, Department of Molecular Virology and Microbiology, Baylor College of Medicine, Mail Stop BCM-385, One Baylor Plaza, Houston, TX 77030, USA. Tel.: +1 713 798 8474; Fax: +1 713 798 3586; E-mail: pling@bcm.tmc.edu or DB Bloch, Department of Medicine, Harvard Medical School and the Center for Immunology and Inflammatory Diseases, Massachusetts General Hospital-East, CNY 149 13th Street, Charlestown, MA 02129, USA. Tel: +1 617 726 3780; Fax: +1 617 726 5651; E-mail: bloch@helix.mgh.harvard.edu

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Introduction

Epstein–Barr virus (EBV) is a causative agent or cofactor in the etiology of several human malignancies including endemic Burkitt's lymphoma, nasopharyngeal carcinoma, some forms of Hodgkin's disease, and lymphomas in immunosuppressed patients (Crawford, 2001; Rickinson and Kieff, 2001). *In vitro*, the virus establishes a latent infection in human B cells and has an intrinsic ability to immortalize these cells through expression of several latent cycle gene products (Bornkamm and Hammerschmidt, 2001). The functions of some of these proteins have already been elucidated. EBNA2 is a transcriptional activating protein that controls viral latent and cellular gene expression via mimicry of cellular Notch signaling pathways (Bornkamm and Hammerschmidt, 2001; Kieff and Rickinson, 2001). Latent membrane protein 1 (LMP-1) functions through interaction with tumor necrosis-associated factors (TRAFs) and resembles a constitutively active CD40 receptor (Bornkamm and Hammerschmidt, 2001; Kieff and Rickinson, 2001). LMP2A operates through B-cell-receptor signaling pathways by its association with lyn and syk (Bornkamm and Hammerschmidt, 2001; Kieff and Rickinson, 2001). In contrast, the mechanistic contributions of EBNA-LP to EBV-induced immortalization remain less well characterized.

EBNA-LP is an unusual protein composed of 22 and 44 amino-acid repeats derived from the W1 and W2 exons found in the large internal repeated region in the virus (IR1) and two unique exons known as Y1 and Y2 (Sample *et al*, 1986; Speck *et al*, 1986). Illustrations of the EBNA-LP coding region relative to the rest of the EBV genome, and of the structure of the EBNA-LP transcript, can be found in Figure S1A. Although EBNA-LP localizes predominantly in the nucleus, the distribution of EBNA-LP within the nucleus is variable. During early infection in B cells and following transient or constitutive expression of EBNA-LP in type I Burkitt's lymphoma cell lines (Rickinson and Kieff, 2001), EBNA-LP is distributed diffusely throughout the nucleus (Szekely *et al*, 1995b, 1996; Nitsche *et al*, 1997). In contrast, in established LCLs, EBNA-LP localizes to promyelocytic leukemia nuclear bodies (PML NBs) (Szekely *et al*, 1995b, 1996).

EBNA-LP function has been investigated using genetic and cell-based assays. A mutant EBV, with a deletion of the carboxy-terminal 45 amino-acid residues encoded by Y1 and Y2, immortalized cells only in the presence of feeder cells (Hammerschmidt and Sugden, 1989; Mannick *et al*, 1991). Once established, however, these cell lines did not differ from wild-type (wt) virus-immortalized cells in phenotype or growth properties (Allan *et al*, 1992). More recent studies found that EBNA-LP stimulates EBNA2-mediated activation of viral latent membrane proteins 1 and 2B (LMP-1 and LMP2B) as well as the C promoter (Cp), a major promoter of latent transcription in immortalized cells (Figure S1A) (Harada and Kieff, 1997; Nitsche *et al*, 1997; Peng *et al*, 2005). Several nonhuman primate lymphocryptoviruses (LCVs) also encode EBNA-LP homologs

and the ability to coactivate EBNA2 is conserved (Peng *et al*, 2000a).

The functional domains in the W1W2 repeats have been identified (Peng *et al*, 2000b; McCann *et al*, 2001). EBNA-LP has a bipartite nuclear localization signal (NLS), which, with a region known as conserved region 3 (CR3), is critical for coactivation function (Peng *et al*, 2000b; McCann *et al*, 2001; Yokoyama *et al*, 2001). At least two copies of the W1W2 repeats are required for coactivation function. The Y1Y2 domains are not needed for this activity (Harada and Kieff, 1997; Nitsche *et al*, 1997; Peng *et al*, 2000b).

Host cell proteins that have been reported to interact with EBNA-LP include pRb, p53, hsp72/hsc73, hsp27, Hax-1, ERR1, p14ARF, DNA-Pkcs, α-tubulin, β-tubulin, prolyl-4-hydroxylase, and HA95 (Jiang *et al*, 1991; Szekely *et al*, 1993, 1995a; Mannick *et al*, 1995; Kitay and Rowe, 1996; Kawaguchi *et al*, 2000; Dufva *et al*, 2001; Han *et al*, 2001, 2002; Igarashi *et al*, 2003; Kashuba *et al*, 2003). So far, however, no correlation has been made between EBNA-LP association with these factors and the ability of EBNA-LP to coactivate EBNA2. Based on the observation that EBNA-LP associated with PML NBs in established LCLs (Szekely *et al*, 1996), we hypothesized that proteins found in these structures might be candidate cofactors for, or mediators of, EBNA-LP function.

The PML NB is a cellular structure that appears to be involved in the pathogenesis of a variety of human diseases including acute promyelocytic leukemia (APL) and viral infections (Sternsdorf *et al*, 1997; Hodges *et al*, 1998; Melnick and Licht, 1999; Pandolfi, 2001). In addition, components of PML NBs, including Sp100, PML, and Sp140, are the targets of autoantibodies in the serum of patients with the autoimmune disease primary biliary cirrhosis (PBC) (Szostecki *et al*, 1987, 1990; Sternsdorf *et al*, 1995; Bloch *et al*, 1996). Proteins that localize to PML NBs include factors involved in gene transcription (PML, CBP, Sp100, heterochromatin protein 1α (HP1α), Sp110, and Sp140), in genomic stability (BLM, MRE11, and p95), in cell-cycle regulation (p53 and pRb), and in apoptosis (PML and Daxx) (Lehming *et al*, 1998; Bloch *et al*, 1999, 2000; Doucas *et al*, 1999; Ishov *et al*, 1999; Guo *et al*, 2000; Li *et al*, 2000; Nicewonger *et al*, 2004). The presence of this diverse group of proteins within PML NBs suggests that this structure may affect or regulate a wide variety of cellular processes.

Herpesviruses interact with and modify PML NBs. Human cytomegalovirus (CMV) genomes initially localize to PML NBs and the CMV Immediate-Early protein 1 (IE1) displaces Sp100 and PML from these structures (Korioth *et al*, 1996; Ahn and Hayward, 1997). Herpes simplex virus (HSV)-1 Immediate-Early Protein Zero (ICP0) disrupts PML NBs by inducing proteosome-dependent degradation of PML and Sp100 (Everett, 2001; Hagglund and Roizman, 2004). During the course of lytic EBV infection, Sp100 and PML are sequentially displaced from PML NBs (Bell *et al*, 2000; Adamson and Kenney, 2001). During viral productive cycles, interactions between viral and PML NB proteins may facilitate viral DNA transcription and replication. Alternatively, disruption of PML NBs may circumvent or disable innate cellular defenses. In this study, we provide evidence that EBNA-LP coactivates EBNA2 through binding Sp100 and displacing it and HP1α from PML NBs.

Results

EBNA-LP displaces Sp100 from PML NBs

To examine the effects of EBNA-LP on the cellular location of components of the PML NB, EBNA-LP was transfected into Hep-2 cells, and cells were stained with mouse anti-EBNA-LP and rabbit anti-Sp100. In these studies, we used EBNA-LP isoforms composed of two W1W2 repeats. This is the minimal isoform of EBNA-LP compatible with B-cell transformation and coactivation (Nitsche *et al*, 1997; Yoo *et al*, 1997; Peng *et al*, 2000b). EBNA-LP expression displaced Sp100 from PML NBs (Figure 1, panel I, A–C). In contrast, an EBNA-LP mutant protein lacking residues from CR3 (ΔCR3LP), which is unable to coactivate, did not displace Sp100 from PML NBs (Figure 1, panel I, D–F). Other previously characterized EBNA-LP mutants, which retain the ability to coactivate, including EBNA-LP PGP13/79AAA (mutCR1aLP) (Peng *et al*, 2000b), also retain the ability to displace Sp100 from PML NBs (data not shown). An EBNA-LP polypeptide containing only a single W1W2 domain, which localizes to the cell cytoplasm and not the nucleus, did not displace Sp100 from PML NBs (data not shown).

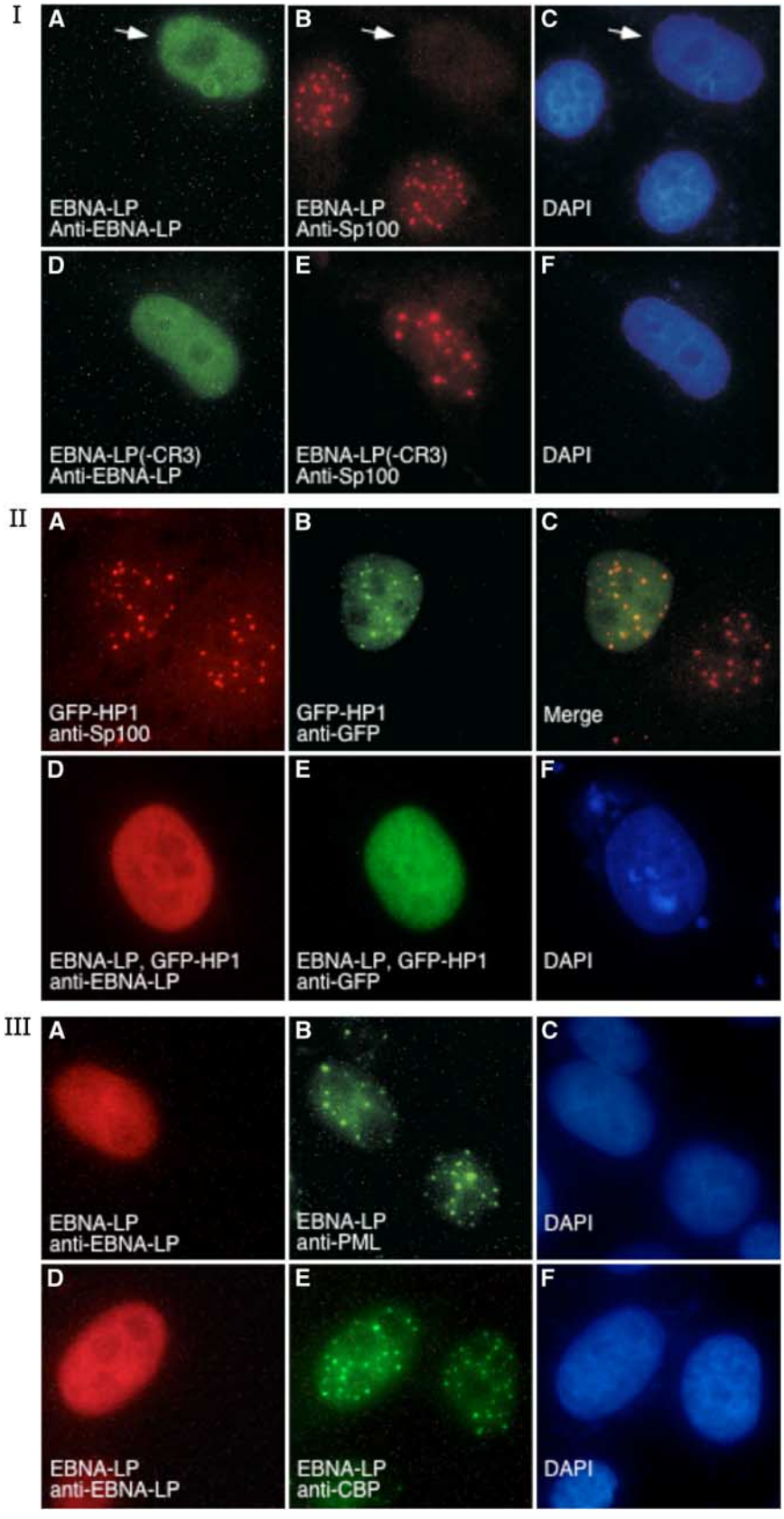
Previous investigators demonstrated that HP1α interacts with PML NB component Sp100. However, because HP1α localizes to heterochromatin, centromeres, as well as PML NBs, we found it difficult to determine the effect of EBNA-LP on endogenous PML NB-associated HP1α. Hayakawa *et al* (2003) demonstrated that fluorescent protein–HP1α fusion protein localizes almost exclusively to PML NBs in interphase cells. We transfected GFP–HP1α into Hep2 cells and confirmed that the fusion protein localizes to PML NBs (Figure 1, panel II, A–C). Coexpression of EBNA-LP with GFP–HP1α resulted in displacement of GFP–HP1α from PML NBs (Figure 1, panel II, D–F).

In contrast to the effects of EBNA-LP on Sp100 and HP1α, the cellular location of PML was not affected by EBNA-LP (Figure 1, panel III, A–C). Similarly, the cellular location of CBP, which is recruited to PML NBs by PML, was also unaffected by EBNA-LP (Figure 1, panel III, D–F). These results show that EBNA-LP selectively modifies PML NBs. To determine the effect of EBNA-LP expression on PML NBs in a more physiologically relevant cell line, EBNA-LP was

Figure 1 Panel I: EBNA-LP, but not EBNA-LP ΔCR3, displaces Sp100 from PML NBs. Expression of EBNA-LP (green, A) in Hep-2 cells resulted in localization of the protein in a diffuse nuclear staining pattern. EBNA-LP displaced Sp100 (red, B) from PML NBs. In contrast, expression of a mutant EBNA-LP, which lacked the CR3 domain (green, D), did not alter the cellular location of Sp100 (red, E). The white arrow in (A–C) points to the EBNA-LP-expressing cell. DAPI staining indicates the location of cell nuclei in (C) and (F). Panel II: EBNA-LP displaces GFP–HP1α from PML NBs. After transfection of Hep-2 cells with a plasmid encoding GFP–HP1α, staining for Sp100 (red, A) and GFP–HP1α (green, B) revealed localization of GFP–HP1α in PML NBs (overlap is shown in yellow in panel C). After expression of EBNA-LP (red, D) and GFP–HP1α in Hep-2 cells, GFP–HP1α did not localize to PML NBs, but was instead distributed diffusely throughout the nucleus (green, E). DAPI staining indicates the location of cell nucleus in (F). Panel III: EBNA-LP did not alter the NB location of PML or CBP. EBNA-LP expression in Hep-2 cells (red, A and D) did not alter the PML NB location of endogenous PML (green B) and CBP (green, E). DAPI staining indicates the location of cell nuclei in (C) and (F).

transiently expressed in EBV-negative DG75 B cells. EBNA-LP displaced Sp100, but not PML, from PML NBs in these cells (Figure S2 A–F).

To determine if EBNA-LP induced Sp100 degradation, rather than displacement from PML NBs, the level of Sp100 was measured in control DG75 B cells and cell lines



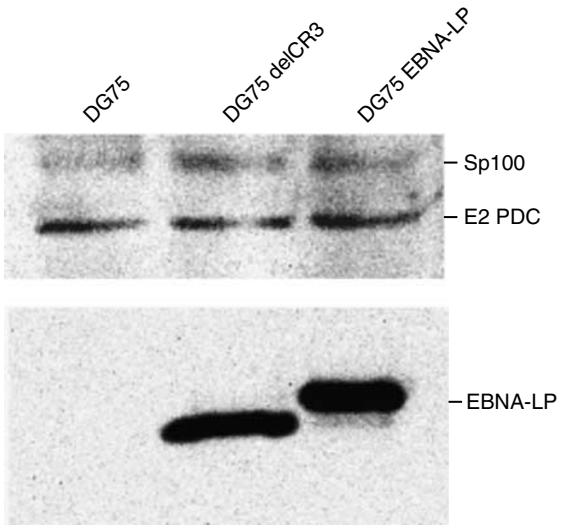


Figure 2 Immunoblot of DG75 B cells and DG75 B cells constitutively expressing EBNA-LP or ΔCR3LP (DG75 delCR3). Antibodies in serum from PBC patient K142 reacted with Sp100 and PBC autoantigen E2 pyruvate dehydrogenase complex (E2 PDC). Mouse anti-EBNA-LP was used to detect EBNA-LP and ΔCR3LP. There was no difference in the level of Sp100 in the three cell lines.

constitutively expressing wt EBNA-LP or ΔCR3 EBNA-LP. There was no difference in the level of Sp100 in these cell lines, indicating that EBNA-LP does not mediate Sp100 destruction (Figure 2).

EBNA-LP interacts with Sp100

To determine whether EBNA-LP-induced displacement of Sp100 from PML NBs was mediated through interactions with PML NB-associated proteins, we overexpressed EBNA-LP with several proteins including Sp100, Daxx, and PML. In these studies, eukaryotic expression vectors encoding the HA epitope fused to Sp100, Daxx, or PML were expressed in EBV-negative Burkitt's lymphoma cells with a Flag epitope fused to EBNA-LP (EBNA-LP-Flag). Cell lysates were incubated with anti-Flag epitope antibodies directed against EBNA-LP-Flag or anti-HA epitope antibodies directed against HA fused to PML, Daxx, or Sp100, and the immunoprecipitated (IP) proteins were assayed by Western blot. EBNA2-HA and EBNA-LP-Flag, which do not interact in this assay (Peng *et al*, 2004, 2005), were used as a negative control. The results showed that EBNA-LP-Flag was able to co-precipitate Sp100 but not Daxx, PML, or EBNA2 (Figure S3).

To determine the specificity of the EBNA-LP–Sp100 interaction, we tested whether the coactivation-deficient mutant ΔCR3LP, which was unable to displace Sp100 from PML NBs, was able to form complexes with Sp100. Sp100-HA was able to co-precipitate EBNA-LP and the coactivation functional mutCR1aLP (Figure 3A, lanes 1 and 7), but not ΔCR3LP (Figure 3A, lane 4). In the reciprocal experiment, ΔCR3LP-Flag was unable to precipitate Sp100-HA (Figure 3A, lane 5). In contrast, both wt and mutCR1aLP co-precipitated Sp100 (Figure 3A, lanes 2 and 8).

To approximate more physiological conditions, we asked whether EBNA-LP and Sp100 associated with each other in EBV-immortalized cells as well as cell lines that constitutively expressed EBNA-LP-Flag or ΔCR3LP-Flag. Coimmunoprecipitation analysis demonstrated that endogenous Sp100 was present in complexes with EBNA-LP in IB4 and MHK EBV-immortalized cell lines. We also detected EBNA-LP interactions with Sp100 in B-cell lines that constitutively express wt EBNA-LP, but not in B-cell lines that constitutively express the ΔCR3 mutant EBNA-LP. These results are presented in Figure S4.

EBNA-LP interacts with the PML NB localization domain of Sp100

We used coimmunoprecipitation assays to identify the Sp100 domain responsible for interaction with EBNA-LP. A panel of Sp100 deletion mutants was constructed that spanned the Sp100 polypeptide (Figure 3B). An SV40 NLS was added to Δ408–480 Sp100 because this deletion removes the endogenous Sp100 NLS (Sternsdorf *et al*, 1999). Some of the deletions were designed to encompass previously described Sp100 functional domains (e.g., homodimerization/PML NB targeting domain, HP1α-binding domain) (Figure 3B). All of the deletion mutants were expressed in EBV-negative Burkitt's lymphoma cells (Figure S5D) and localized to the nucleus (data not shown). The results show that deletion of Sp100 amino-acid residues 3–152, but not other deletions (i.e., 153–286, 287–333, 333–407, and 408–480), abolished the ability of Sp100 to interact with EBNA-LP in coimmunoprecipitation assays (Figure 3C). Notably, amino-acid residues 3–152 constitute a previously described region in Sp100 that confers dimerization and localization to PML NBs (Sternsdorf *et al*, 1999; Negorev *et al*, 2001). The location of the Sp100 domain that mediates interaction with EBNA-LP was confirmed using mammalian two-hybrid assays (Figure S5).

Subcellular localization of Sp100 during EBV infection of primary B cells

To investigate the effect of EBV on the location of Sp100 in primary B cells, we infected freshly isolated cells with EBV. Beginning 48 h after infection, we fixed and stained aliquots of cells with anti-EBNA-LP (to identify infected cells) and either anti-Sp100 or anti-PML antibodies. At 48 h after infection, Sp100 was displaced from PML NBs in ~90% of EBNA-LP-positive cells (Figure 4A–C). In contrast, the location of PML was not altered in EBNA-LP-positive cells (Figure 4D–F). The percentage of EBNA-LP-positive cells with Sp100 displaced from PML NBs decreased with time (72 h, 51%; 96 h, 20%; 120 h, 4%). At 120 h after infection, both EBNA-LP and Sp100 localized to NBs in nearly all of the cells (Figure 4G–I). Note that in LCLs, EBNA-LP and Sp100 also localized to NBs (Figure 4J–L). These results show that during acute EBV infection, Sp100, but not PML, is displaced from PML NBs. However, over time, both EBNA-LP and Sp100 localize to NBs. Sp100 displacement from PML NBs in primary B cells appears to be specifically induced by EBV. B-cell mitogens such as *Staphylococcus aureus* Cowen strain I (SAC) increased the number and size of Sp100-containing PML NBs, but did not displace Sp100 from these structures (data not shown).

An Sp100 mutant lacking the PML NB localization domain coactivates EBNA2 in the absence of EBNA-LP

As EBNA-LP interacts with Sp100 and displaces Sp100 from PML NBs in EBNA-LP-transfected cells, we tested the possibility that Sp100 proteins lacking a PML NB-targeting domain or localizing outside of these structures (due to overexpres-

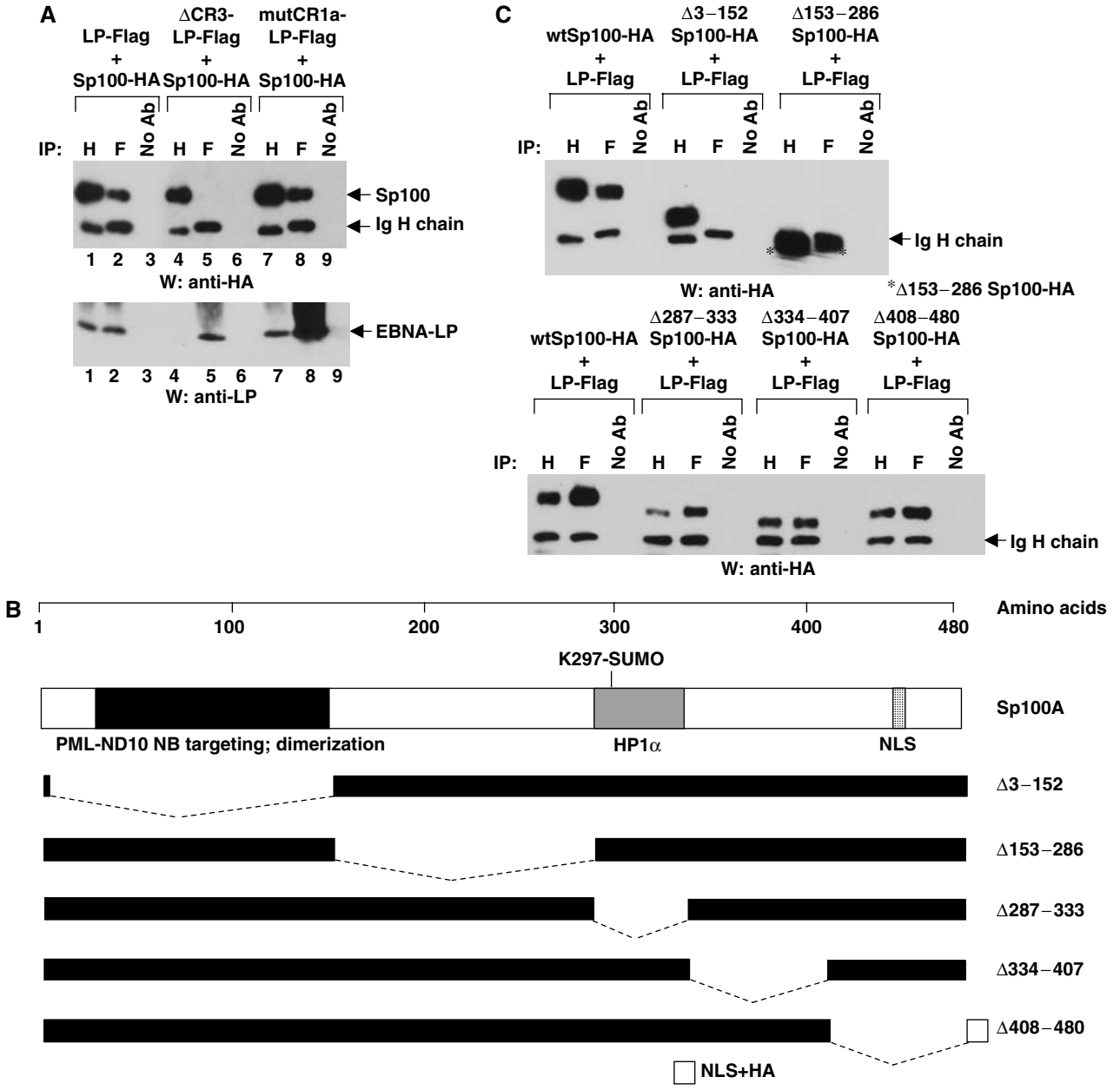


Figure 3 (**A**) EBNA-LP interacts with Sp100. DG75 cells cotransfected with Sp100-HA and LP-Flag, or Sp100-HA and ΔCR3LP-Flag, or Sp100-HA and mutCR1aLP-Flag were lysed and precipitated with anti-HA or anti-Flag antibodies. The extracts were divided into equal parts, resolved by SDS–PAGE, and the proteins were detected by immunoblotting with anti-HA (top panel) or anti-EBNA-LP (bottom panel) antibodies. The migration of Sp100, EBNA-LP, and immunoglobulin heavy (Ig H) chain from the primary antibody used in the IP is indicated. As a control, each extract was also treated with Staph A beads alone (no Ab). The coexpressed proteins contained in each extract are indicated above the panel. Precipitation with anti-HA or anti-Flag antibody is indicated above each lane. (**B**) Schematic of Sp100. Functional domains in Sp100 include amino-acid residues 1–152 (PML NB targeting domain and Sp100 homodimerization region), 287–333 (HP1α interaction domain and SUMO modification site), and 444–450 (nuclear localization sequence). Five Sp100 deletion mutants were used in coimmunoprecipitation and functional studies as indicated. (**C**) EBNA-LP interacts with the PML NB-targeting domain in Sp100. Lysates from cells cotransfected with EBNA-LP-Flag and one of each of the Sp100-HA deletion mutants were immunoprecipitated with anti-HA (H) or anti-Flag (F) antibodies and the resulting precipitates were probed for Sp100 using anti-HA antibodies. The Δ153–286 Sp100 mutant migrated just below the Ig H chain and is designated by asterisks. Each cell extract was also mock-precipitated as a control (no Ab).

sion (Negorev *et al*, 2001)) might be able to coactivate EBNA2 in the absence of EBNA-LP. In transient cotransfection experiments in Eli-BL cells, we found that both wtSp100 and, to a greater extent, Δ3–152 Sp100 were able to coactivate EBNA2 induction of LMP-1 in the absence of EBNA-LP (Figure 5A, top panel, lanes 3–5 and 6–8, respectively). We also tested the other Sp100 deletion mutants shown in Figure 3B for EBNA-LP-independent coactivation. Overexpression of one of the mutants, Δ287–333 Sp100, which lacks the interaction domain for binding HP1α, was unable to coactivate EBNA2 in these assays (Figure 5A, top panel, lanes 9–11). In addition, neither PML (Figure 5B, top panel, lane 7) nor Daxx (data not shown) was able to coactivate EBNA2 in these assays. These results show that the coactivation function of EBNA-LP can

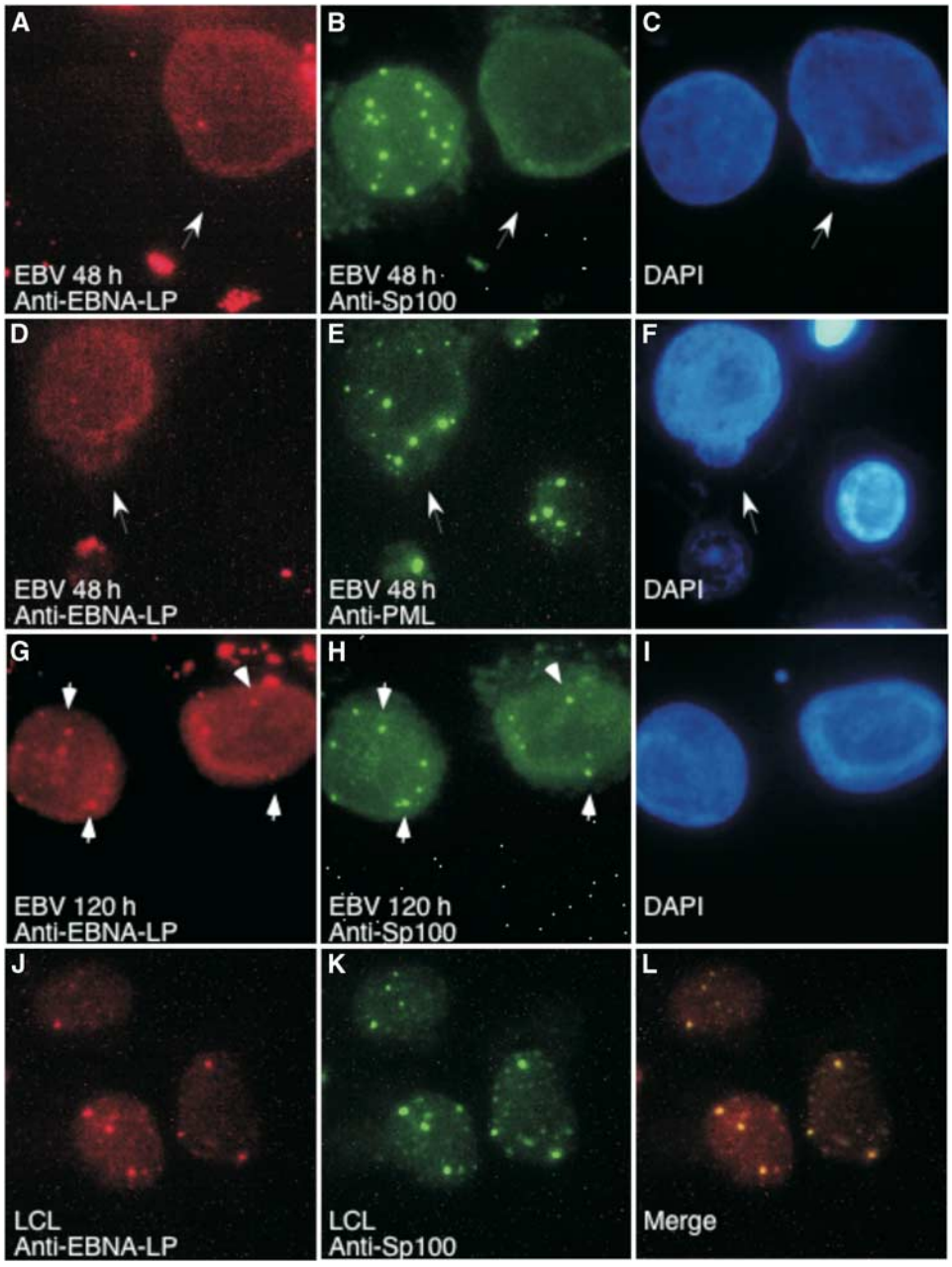


Figure 4 Sp100, but not PML, is displaced from PML NBs following EBV infection of B lymphocytes. At 48 h after EBV infection, Sp100 (green, **B**) was displaced from PML NBs in nearly all infected cells. EBV-infected cells were identified by staining with anti-EBNA-LP antibodies (red, **A, D, G, J**). PML (green, **E**) was not displaced from NBs during EBV infection. At 120 h after infection, both Sp100 (green, **H**) and EBNA-LP localized to PML NBs. In LCLs, both Sp100 (green, **K**) and EBNA-LP (red, **J**) localized to NBs. Overlap between Sp100 and EBNA-LP appears yellow in (**L**). White arrows in (A–F) indicate EBV-infected cells. White arrows in (G, H) point to examples of EBNA-LP and Sp100 in NBs. DAPI staining indicates the location of nuclei in (**C, F, I**).

be replaced by overexpression of Sp100 or by expression of mutant Sp100 that is unable to interact with PML NBs. In addition, the HP1α interaction domain in Sp100 is required for coactivation of EBNA2.

The HP1α interaction domain in Sp100 contains a lysine residue (K297) that can be modified by SUMO. To test the possibility that SUMO is required for coactivation of EBNA2, an Sp100 mutant protein (K297R), which is not a target of SUMO modification (Sternsdorf *et al*, 1999), was expressed in Eli-BL cells. Sp100 K297R coactivated EBNA2 to the same extent as, or greater than, wtSp100 and Δ3–152 Sp100, suggesting that SUMO modification of Sp100 is not required for coactivation function (Figure 5C).

To determine whether displacement of Sp100 correlates with increased LMP-1 expression at the single-cell level, we transfected Eli-BL cells with EBNA2 and EBNA-LP. Cells were fixed and stained with anti-Sp100 antiserum and monoclonal anti-LMP-1 antibody. Sp100 was displaced from PML NBs in all cells that expressed LMP-1 (a representative example is shown in Figure 5D).

To consider the possibility that low levels of endogenous EBV latent proteins (e.g., EBNA2 or EBNA-LP) were

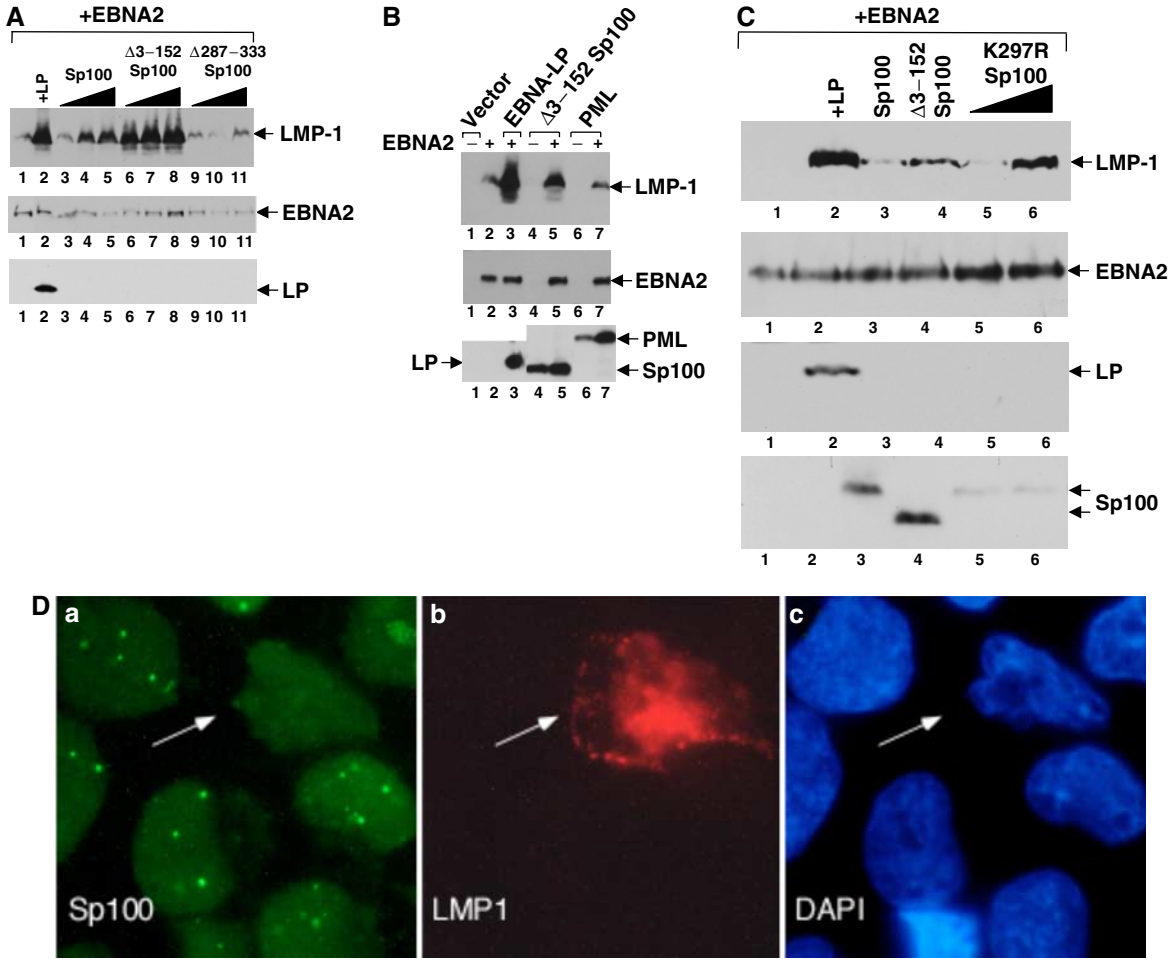


Figure 5 Overexpression of wtSp100 or Δ3–152 Sp100 coactivates EBNA2 in the absence of EBNA-LP. Coactivation of EBNA2 depends on the Sp100–HP1α interaction domain. (**A**) Eli-BL cells were transfected with EBNA2 or EBNA2 and EBNA-LP or EBNA2 and Sp100. Increasing doses of the Sp100 expression plasmids were cotransfected with EBNA2 as indicated. Cell extracts were probed by Western blot for LMP-1 expression (top panel). Levels of EBNA2 and EBNA-LP were detected in these extracts by Western blot using anti-EBNA2 and anti-EBNA-LP antibodies, respectively. (**B**) To examine the ability of another NB component to coactivate LMP-1 expression, PML was cotransfected with EBNA2 and the level of LMP-1 was measured by immunoblot. In the absence of EBNA-LP, PML was unable to enhance EBNA2-induced LMP-1 expression above the level of EBNA2 alone (compare lanes 7 and 2). For comparison, the ability of EBNA-LP and Δ3–152 Sp100 to induce LMP-1 expression is shown (lanes 3 and 5). Successful production of EBNA2, EBNA-LP, Sp100, and PML is demonstrated in the immunoblots below (lower panels). (**C**) Eli-BL cells were transfected with EBNA2 or EBNA2 and EBNA-LP or EBNA2 and various Sp100 mutants. Increasing amounts of the expression plasmid encoding K297R Sp100 were cotransfected with EBNA2 as indicated. Cell extracts were probed by Western blot for LMP-1 expression (top panel). Levels of EBNA2, EBNA-LP, and overexpressed Sp100 proteins were detected in these extracts by Western blot using anti-EBNA2, anti-EBNA-LP, and anti-HA antibodies, respectively, and are shown in the lower panels. (**D**) Immunofluorescence of Eli-BL cells cotransfected with EBNA2 and EBNA-LP were stained with anti-Sp100 (**a**) and anti-LMP-1 (**b**). Sp100 was displaced from PML NBs in all cells that expressed LMP-1. White arrow points to the transfected cell. DAPI staining indicates the location of nuclei in (**c**).

expressed in Eli-BL cells and may cooperate with Sp100 to coactivate EBNA2, we repeated coactivation experiments in the EBV-negative B-cell line DG75. Cotransfection of EBNA2 with an EBNA2-responsive luciferase reporter plasmid (Peng *et al*, 2000b) resulted in approximately 10-fold activation of the reporter. Expression of both EBNA2 and EBNA-LP further stimulated reporter activity 7–8-fold above that obtained with EBNA2 alone, in agreement with our previously published observations (Peng *et al*, 2000b). As predicted from data shown in Figure 5A, expression of EBNA2 with Δ3–152 Sp100 or wtSp100 increased luciferase activity five- and two-fold (respectively) above EBNA2 alone (Figure 6A). Overexpression of Δ3–152 Sp100 or wtSp100 without EBNA2 had no transcription-activating function (Figure 6A). Expression levels of transfected proteins in these experiments are shown in Figure 6B.

Our previous studies showed that EBNA-LP preferentially coactivates EBNA2 stimulation of the *LMP-1* gene, but not the *LMP2A* gene (Peng *et al*, 2005). To determine whether EBNA-LP-independent coactivation by Sp100 retained the gene specificity of EBNA-LP, real-time reverse transcription (RT)–PCR was employed to quantitate levels of the viral LMP-1 and LMP2A transcripts in transfected cells. The results indicated that, as with EBNA-LP, Δ3–152 Sp100 coactivated EBNA2 induction of the *LMP-1*, but not the *LMP2A*, gene (Table I).

Discussion

In this study, we demonstrated that the PML NB-associated protein Sp100 is an important cellular cofactor for EBNA-LP coactivation function. Evidence for this conclusion is based on the ability of EBNA-LP, but not a coactivation mutant

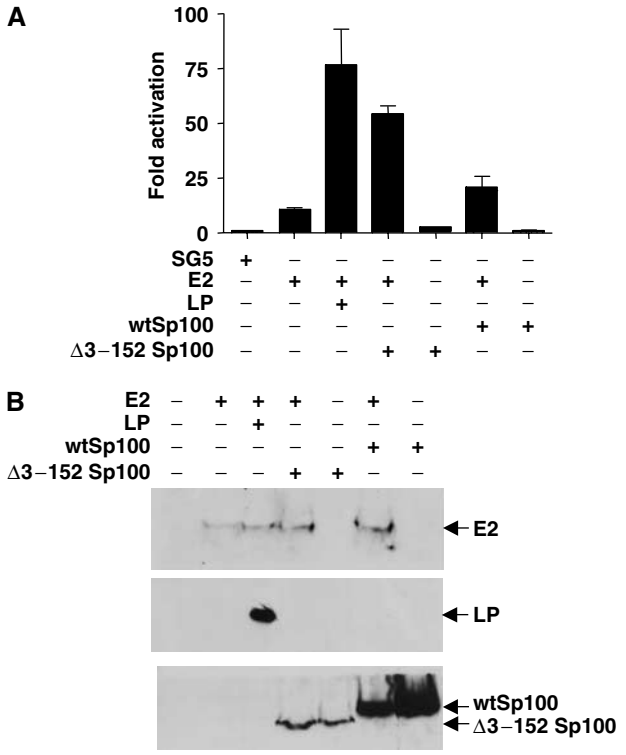


Figure 6 EBNA-LP independent coactivation of EBNA2 by Sp100 in EBV-negative cells. (**A**) DG75 cells were transfected with vector alone (SG5), EBNA2 (E2), EBNA-LP (LP), wtSp100, or an Sp100 deletion mutant (Δ3–152 Sp100) as indicated below the graph. Luciferase activity is shown as fold activation above control. Results are an average of three independent experiments with standard errors. (**B**) Western blots of cell extracts used for the luciferase assays in (A). The top panel shows extracts from each transfection probed for EBNA2 (E2). The middle and bottom panels are identical blots probed with anti-EBNA-LP and anti-HA antibodies, respectively.

Table I Real-time RT–PCR detection of LMP-1 and LMP2A

Sample[a]	Transcript	Relative fold induction, as compared to EBNA2-only sample[b]
EBNA2	LMP-1	1.00±0.18
EBNA2 + EBNA-LP	LMP-1	3.42±0.061
EBNA2 + Δ3–152Sp100	LMP-1	3.24±0.058
EBNA2	LMP2A	1.00±0.19
EBNA2 + EBNA-LP	LMP2A	0.846±0.20
EBNA2 + Δ3–152Sp100	LMP2A	1.07±0.083

[a]RNA from cells transfected with the indicated plasmids was reverse transcribed and the cDNA was amplified by real-time PCR as described in Materials and methods.
[b]Fold induction was calculated using the AB User Bulletin 2 calibrator method for relative quantitation; relative transcript induction was normalized to actin and then expressed as a ratio to the EBNA2-only sample. Numbers represent an average of duplicate samples and are representative of multiple experiments.

containing a deletion in CR3, to bind Sp100 and cause its displacement from PML NBs (Figure 1, panel I and Figure 3A). EBNA-LP facilitates displacement of Sp100 by interacting with the PML NB localization domain, possibly masking or altering its ability to form contacts with factors that target Sp100 to NBs (Figure 3C). Expression of a mutant Sp100 that was unable to associate with PML NBs coactivated EBNA2 in the absence of EBNA-LP (Figures 5A and B and 6), suggesting that displacement of Sp100 from PML NBs is an important step for EBNA2 coactivation. Finally, the interaction between Sp100 and HP1α is important for coactivation of EBNA2 because a mutant form of Sp100 lacking the HP1α interaction domain failed to coactivate EBNA2 (Figure 5A).

Several DNA viruses have been shown to modulate PML NB-associated proteins (Everett, 2001). For example, HSV ICP0 induces destruction of PML NBs and degradation of both PML and Sp100 (Chelbi-Alix and de The, 1999; Gu and Roizman, 2003). CMV IE1 also disrupts PML NBs, but does not induce Sp100 degradation (Korioth *et al*, 1996; Ahn and Hayward, 1997). Adenovirus E4orf3 and the Papilloma virus minor capsid protein L2 associate with and induce release of PML NB-associated proteins (Everett, 2001). In the case of CMV and HSV, viral genomes appear to associate at or near PML NBs shortly after infection. Disruption of PML NBs and subsequent release or degradation of PML NB-associated proteins have been suggested to facilitate more efficient viral gene expression, particularly during the course of early events following infection (Everett, 2001; Hagglund and Roizman, 2004).

Both HP1α and Sp100 have been shown to possess transcriptional repressor activity (Lehming *et al*, 1998; Seeler *et al*, 1998, 2001). HP1α has a role in the organization of chromatin and promotes silencing of euchromatic genes (Singh and Georgatos, 2002). Sp100, when tethered to the promoter region of a reporter gene, represses gene expression (Lehming *et al*, 1998; Seeler *et al*, 1998; Bloch *et al*, 1999). Sp100 has also recently been shown to inhibit the function of the transcriptional activator ETS1 (Yordy *et al*, 2004). If EBV genomes associate with PML NBs, then some viral promoters, especially the LMP-1 promoter, may be subject to the repressive effects of these proteins. EBNA-LP might reverse repression by displacing these factors from the EBV genome, creating a more transcriptionally favorable environment for EBNA2 to promote gene expression. Although Bell *et al* (2000) previously showed that EBV genomes do not associate with PML NBs in latently infected cells, these studies were performed in a hybrid cell line (D98/HR1) derived from a fusion between epithelial cells and Burkitt's lymphoma cells. D98/HR1 cells harbor the P3HR1 variant that lacks EBNA2 and part of the *EBNA-LP* gene. Whether this accurately reflects the EBV genome location in all type I EBV-positive Burkitt's lymphoma cells (e.g., Eli-BL) remains to be established.

Although HP1α and Sp100 are generally considered inhibitors of gene expression, these proteins may also have a role in increasing gene expression. Xie *et al* (1993) demonstrated that Sp100 contains a cryptic activation domain. Moller *et al* (2003) showed that Sp100 was important for the stimulatory effect of homeodomain-interacting protein kinase-2 on p53-dependent gene expression. HP1α, in its role as organizer of chromatin structure, may enhance transcription of genes that lie within heterochromatic regions (Eissenberg and Elgin, 2000). If EBV genomes do not localize to PML NBs, then HP1α may associate with the viral genomes at other locations in the nucleus. EBNA-LP-mediated displacement of Sp100 from PML NBs may permit Sp100 to bind to EBV genomes via interaction with HP1α. Together, Sp100 and HP1α may enhance viral gene expression.

The interaction between EBNA-LP and Sp100 may increase gene expression through mechanisms other than enhanced

gene transcription. A recent study showed that Sp110b, a component of the PML NB and a member of the Sp100 family of proteins, interacts with the EBV lytic cycle protein SM (Nicewonger *et al*, 2004). The EBV SM–Sp110b interaction resulted in increased stability of SM-regulated viral transcripts. It is possible that the EBNA-LP–Sp100 interaction, like that of EBV SM–Sp110b, may also alter gene expression through post-transcriptional mechanisms.

HP1α has a complex pattern of intracellular localization, depending on the presence or absence of a variety of interacting proteins and the phase of the cell cycle (Everett *et al*, 1999; Hayakawa *et al*, 2003). As endogenous HP1α localizes to several different cellular domains, including heterochromatin, centromeres, as well as PML NBs, we were unable to determine the effect of EBNA-LP on the PML NB localization of endogenous HP1α. However, Hayakawa *et al* showed, and we have confirmed in this study, that expression of a GFP–HP1α fusion protein results in localization of HP1α to PML NBs in nearly all interphase cells. Coexpression of EBNA-LP and GFP–HP1α resulted in displacement of GFP–HP1α from PML NBs. In view of the previous studies showing that HP1α interacts with Sp100, and our findings that EBNA-LP displaces Sp100 and GFP–HP1α from PML NBs, it seems likely that EBNA-LP also alters the cellular location of endogenous, PML NB-associated HP1α.

Similar to the other herpesvirus immediate early proteins, EBNA-LP coactivation function is likely to play an important role during early events of infection. EBNA-LP and EBNA2 are the earliest latent cycle proteins detected following infection of primary B cells. It is likely that EBV genomes are subject to similar host cell modifications encountered by other herpesviruses following infection. EBNA-LP may mitigate transcriptional barriers that prevent efficient expression of viral latent genes important for establishing latent infection. Although EBNA-LP and Sp100 are distributed diffusely throughout the nucleus during early infection, EBNA-LP and Sp100 localize to PML NBs in established lymphoblastoid cell lines immortalized by EBV. It is possible that other viral latent cycle proteins may act to regulate EBNA-LP–Sp100 activity as latent infection becomes established. This may be particularly important because latent viral genes like *LMP-1* are cytostatic when overexpressed (Floettmann *et al*, 1996). Inhibition of EBNA-LP activity would prevent overly robust LMP-1 expression, which would negatively affect LCL growth. Thus, EBNA -LP may play an important role during early EBV infection, but may not be required for maintenance of (or in fact may be detrimental to) immortalization in established LCLs.

Identification of cellular cofactors that mediate EBV latent protein functions has been a central question in EBV biology. Our data are the first to show that modulation of PML NBs might be required for establishment of nonproductive or latent herpesvirus infections. In this study, we used EBNA-LP isoforms with only two W1W2 repeats. Most EBV strains synthesize EBNA-LP polypeptides with several W1W2 repeats. The existence of multiple Sp100 interaction domains in a single EBNA-LP polypeptide may make it particularly adept at binding and displacing Sp100 from PML NBs. In contrast to CMV IE1 or HSV ICP0; EBNA-LP does not appear to displace PML from NBs, but instead seems to selectively displace Sp100 and HP1α from these structures. Thus, EBNA-LP appears to modulate PML NBs by a more subtle mechanism than observed for ICP0 and IE1. This may be important for EBV, as it tends to favor coexistence with the host cell rather than host cell destruction. At present it is unclear why EBNA-LP and IE1 redistribute Sp100, while HSV ICP0 induces Sp100 degradation.

Historically, the function of numerous cellular proteins has been elucidated through investigation of their interactions with viral proteins. Induction of LMP-1 expression in Eli-BL cells, which is potentiated through interactions between EBNA-LP and Sp100, provides a unique system to unravel how the cellular Sp100 protein regulates gene expression and to identify the cellular genes that are targeted for regulation by this protein.

Materials and methods

Cell culture, cell lines, transfections, and plasmids

Eli-BL, an EBV type I Burkitt's lymphoma line, DG75, an EBV-negative Burkitt's cell line, and Hep-2 cells were maintained and transfected as described previously (Bloch *et al*, 1999; Peng *et al*, 2000b, 2005). To generate cell lines constitutively expressing EBNA-LP, we cotransfected plasmids pRSP438 or pJT125 with a plasmid expressing the puromycin-*N*-acetyl-transferase gene (pGK3PURO), and cell clones emerging under puromycin selection were screened for EBNA-LP expression. Plasmids used in this study were generated using standard procedures (see Supplementary data for details).

B-cell isolation and EBV infection

Primary human B cells were isolated from healthy donor buffy coats (Gulf coast regional blood center). The buffy coats were diluted 1:2 in PBS and the lymphocytes were purified on Ficoll gradients. B cells were selected using CD19 magnetic beads (Miltenyi). B cells (1×10^6) were incubated with 10 ml of virus-containing supernatant derived from B95-8 cells as described (Ling and Hulls, 2005). The infected cells were incubated at 37°C. Aliquots of infected cells were harvested at the indicated time-points and prepared for indirect immunofluorescence as described below.

RNA extraction and RT–PCR

RNA was prepared from transfected cells using the TRIZOL (Invitrogen) extraction method. Complementary DNA was prepared from 0.5–1 μg of RNA using AMV reverse transcriptase (Invitrogen). Subsequent PCR reactions were performed using oligonucleotide primers as described previously (Peng *et al*, 2005).

Western blots, IPs, and indirect immunofluorescence

Western blotting was carried out as described previously (Peng *et al*, 2000b, 2005). Serum from patient K142 with PBC contains antibodies directed against Sp100 and PBC autoantigen E2 pyruvate dehydrogenase complex (Bloch *et al*, 1999). For IPs, transfected cells were lysed in either RIPA buffer or a 1% NP40 buffer (10 mM Tris-Cl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 3% glycerol, 1 mM phenylmethylsulfonyl fluoride, 5 mg/ml leupeptin, 10 mg/ml aprotinin). Transfected cell lysates were incubated with a primary antibody at 4°C overnight, followed by incubation with Staph A sepharose beads (Pierce) for 1 h at room temperature. The beads were washed in IP lysis buffer and the bound proteins were solubilized by addition of 2 × Laemmli sample buffer and boiling for 5 min. The proteins were subjected to SDS–PAGE and Western blotting. Indirect immunofluorescence was performed as described previously (Bloch *et al*, 1999).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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