Interferon Regulatory Factor 7 Is Negatively Regulated by the Epstein-Barr Virus Immediate-Early Gene, *BZLF-1*

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Virus infection stimulates potent antiviral responses; specifically, Epstein-Barr virus (EBV) infection induces and activates interferon regulatory factor 7 (IRF-7), which is essential for production of alpha/beta interferons (IFN-α/β) and upregulates expression of Tap-2. Here we present evidence that during cytolytic viral replication the immediate-early EBV protein BZLF-1 counteracts effects of IRF-7 that are central to host antiviral responses. We initiated these studies by examining IRF-7 protein expression in vivo in lesions of hairy leukoplakia (HLP) in which there is abundant EBV replication but the expected inflammatory infiltrate is absent. This absence might predict that factors involved in the antiviral response are absent or inactive. First, we detected significant levels of IRF-7 in the nucleus, as well as in the cytoplasm, of cells in HLP lesions. IRF-7 activity in cell lines during cytolytic viral replication was examined by assay of the IRF-7-responsive promoters, IFN-α4, IFN-β, and Tap-2, as well as of an IFN-stimulated response element (ISRE)-containing reporter construct. These reporter constructs showed consistent reduction of activity during lytic replication. Both endogenous and transiently expressed IRF-7 and EBV BZLF-1 proteins physically associate in cell culture, although BZLF-1 had no effect on the nuclear localization of IRF-7. However, IRF-7-dependent activity of the IFN-α4, IFN-β, and Tap-2 promoters, as well as an ISRE promoter construct, was inhibited by BZLF-1. This inhibition occurred in the absence of other EBV proteins and was independent of IFN signaling. Expression of BZLF-1 also inhibited activation of IRF-7 by double-stranded RNA, as well as the activity of a constitutively active mutant form of IRF-7. Negative regulation of IRF-7 by BZLF-1 required the activation domain but not the DNA-binding domain of BZLF-1. Thus, EBV may subvert cellular antiviral responses and immune detection by blocking the activation of IFN-α4, IFN-β, and Tap-2 by IRF-7 through the medium of BZLF-1 as a negative regulator.

Attachment and entry of herpesviruses during infection evokes a potent initial interferon (IFN) response (10, 21, 52, 55, 66, 72, 74). IFN-αs and IFN-β are cytokines involved in numerous immune interactions during viral infection (reviewed in reference 21), and IFNs are best known for activating IFN-stimulated genes (ISGs), which in turn inhibit viral replication through degradation of viral and cellular RNA and inhibition of protein translation. IFN-α and IFN-β also have important immunomodulatory activities. They enhance major histocompatibility complex (MHC) class I antigen presentation in virally infected cells, thus increasing the susceptibility of infected cells to surveillance and destruction by CD8+ cells. IFN-α and IFN-β also activate and shape the cytotoxic response of natural killer cells, affect dendritic cell maturation and antigen presentation, increase Langerhans cell migration (39), and are involved in the complex regulation of interleukin-12 (IL-12), IL-15, IFN-α, IFN-β, and IFN-γ cytokine responses (32, 78). Thus, signaling from IFN-αs and IFN-β provides the first line of defense against viral infection, as well as fine-tuning of the adaptive immune reaction to viral infection.

The promoters for IFN-αs and IFN-β contain IFN-stimulated response elements (ISREs) which interact with IFN regulatory factors (IRFs) (40). IRFs are transcription factors that play critical roles in ISG expression, cell growth, and differentiation. They interact with ISRE sequences through helix-turn-helix DNA-binding motifs in their amino termini. IRF-7 and IRF-3 are the key regulators of the IFN-α/β responses. Their transcriptional targets include IFN-α4, IFN-β, and other ISGs.

Despite triggering host antiviral responses during virus attachment and entry, herpesvirus replication still proceeds in infected cells (21, 33, 42, 44, 64). Herpes simplex virus (HSV-1) and Kaposi's sarcoma herpesvirus (KSHV) have evolved immune evasion strategies that target IRF-7 responses. Endogenous IRF-7 expression is normally limited to peripheral lymphocytes, dendritic cells, spleen, and lymph nodes (7, 88), but it can be induced by IFN-α and -β, Epstein-Barr virus (EBV) latent membrane protein 1 (LMP-1), and other means (40, 89). IRF-7 is localized to the cytoplasm until it is activated by stimuli such as viral infection, double-stranded RNA (dsRNA) signaling, TLR-3 signaling, or LMP-1 signaling (40, 91). The noncanonical IκB kinases, IKKε and TBK-1, phosphorylate IRF-7 in response to viral infection (71), which leads to either heterodimerization with IRF-3 or homodimerization and nuclear localization where it binds responsive promoters (21, 91). IRF-7 is also important in the activation of the transporter associated with the antigen processing 2 (Tap-2) promoter by LMP-1 (91). This finding is noteworthy because the Tap-2 gene

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product is crucial for the delivery of peptides from the cytoplasm to the lumen of the endoplasmic reticulum, where peptides are loaded onto MHC class I molecules for presentation to cytotoxic T lymphocytes (2). Furthermore, during latent EBV infection Tap-2, along with other MHC antigen-presenting molecules, is upregulated by LMP-1 signaling (31, 60, 69, 77, 91, 94).

Since other herpesviruses have evolved mechanisms to block IFN responses by inhibiting IRF-7 activity, we speculated that EBV also had such a mechanism in the cytolytic cycle. EBV is a gammaherpesvirus that establishes infection initially in the oropharynx and, although latent replication is ultimately the outcome of infection, EBV initially produces a cytolytic infection. A pathological manifestation of permissive EBV infection is oral hairy leukoplakia (HLP), which presents as hyperkeratotic lesions in squamous tongue epithelium that are uniquely characterized by localized viral replication (18, 22, 23, 79). Features of this infected tissue include parakeratosis of the superficial epithelial layer, acanthosis of the mid-stratum spinosum, and ballooning degeneration of koilocyte-like cells (24, 62, 79). Viral lytic cycle EBV proteins are detected in the spinosum of HLP, including BZLF-1, BRLF-1, and BHRF-1 gene products, as well as latent cycle genes such as LMP-1, LMP-2A, LMP-2B, EBV nuclear antigen 1 (EBNA-1), EBNA-2, and EBNA-LP (16, 61–63, 81–84). Importantly, LMP-1 signaling seems intact in HLP lesions since the protein's associated cofactors, such as TRAFs, are found colocalized and downstream targets such as activated NF-κB are detected in the nucleus (83). Lytic viral replication is abundant in the mid- and upper-stratum spinosum of HLP lesions, whereas few lytic or latent viral proteins are detected in the basal epithelial layer (79).

Interestingly, despite high levels of viral replication in HLP, T-helper cells and oral mucosal Langerhans cells are absent (14). Langerhans cells are bone marrow-derived dendritic cells found in the skin and mucosa that serve a critical role in preventing the spread of epithelial infections (8). EBV lytic replication in HLP is associated with decreased Langerhans cell counts; inhibition of viral replication restores these numbers (80). This paucity of inflammatory infiltrate suggests that EBV, like other herpesviruses, has mechanisms to evade antiviral responses perhaps through IRF-7 inhibition.

A candidate EBV gene for the inhibition of IRF-7 activity is *BZLF-1*. The *BZLF-1* gene product (also called Z, Zebra, Zta, and EB-1) is an immediate-early protein that initiates the transcription of viral early gene promoters resulting in the temporal cascade leading to viral replication (9, 73). *BZLF-1* has known immunomodulatory effects: it impedes IFN-γ signaling (51), it is linked to reduced expression of MHC class I cell surface proteins (31), it can cause dispersal of nuclear PML bodies (4), and it interacts with (26) and modulates p65 activity (49), as well as modulating TNF-α signaling (50) and p53 signaling, (95), while increasing ATF2 activation (3). These observations suggest that BZLF-1 can regulate immune responses.

We demonstrate here that EBV utilizes a BZLF-1-mediated mechanism that inhibits the antiviral response of the cell through negative regulation of IRF-7 activity on the IFN-α4, IFN-β, and Tap-2 promoters. This regulation is important for understanding critical aspects of the immune response to EBV lytic replication and in the pathogenesis of the HLP lesion.

MATERIALS AND METHODS

Cell culture. U4A cells lacking Jak-1 and the parental cell line 2fTGH (human fibroblast cell line) were gifts from George Stark (68), and TLR-3-expressing 293 cells were a gift from Tom Maniatis (19). The 293, U4A, 2fTGH, and TLR-3-expressing 293 cell lines were maintained as described previously (19, 20, 68). X50-7 and Raji cell lines were maintained as described previously (47, 59, 67). TLR-3/293 cells were induced with 10 μg of poly(I-C) (Sigma)/ml. Viral reactivation in Raji cells was induced with 5 μg of methotrexate (Immunex, Thousand Oaks, CA)/ml as described previously (17).

Plasmids. pSG-BZLF-1 and the mutants pSG-BZLF-1 ΔTA and pSG-BZLF-1 A185K were gifts from Shannon Kenney and have been described (50). The LMP-1, pcDNA-CD4, EGFP-IRF-7, and Tap-2 CAT plasmids have been described before (91); pISRE-luc construct was purchased from BD Biosciences/ Clontech. BKRF-4 cDNA was PCR amplified from the EBV B95-8 genome and cloned into pCMV2-Flag. The IFN-β pGL3 and IFN-α4 pGL3 promoter constructs and the IRF-7 expression plasmid (pCMV2-Flag plasmid containing wild-type IRF-7A and the aspartic acid substitution mutant, IRF-7D477/D479) were described previously (35, 36) and were gifts from Rongtuan Lin and John Hiscott.

Transfections, reporter assays, and CD4 selection. Cells grown overnight in 12-well plates were transfected with FuGENE reagent (Roche) and 0.1 μg of reporter plasmids and 0.1 μg of expression plasmids unless otherwise noted and 0.025 μg of *Renilla* luciferase plasmid (pRL-TK [Promega]). X50-7 or Raji cells were transfected with 10 μg of plasmid DNA and electroporated as described previously (91). X50-7 cells were nucleoporated using the Amaxa Kit (Gaithersburg, MD): 10^6 cells were suspended in 100 μl of nucleofector solution R, plasmid DNA was added, and electroporation was done by using program T-01. At 24 h after transfection, cells were harvested for luciferase assays, chloramphenicol acetyltransferase (CAT) assays, or CD4 selection. Luciferase assays were performed as described before (58) with dual luciferase assay reagent (Promega); relative light units were normalized to the activity of *Renilla* luciferase used as an internal control. CAT assays were performed with the Promega CAT enzyme assay systems, quantitated by liquid scintillation counting, and normalized to the activity of *Renilla* luciferase. Representative data of three experiments done in triplicate are shown. For the selection of transfected cells, 24 h after nucleofection, samples of transfected cells were collected and pooled after CAT samples were taken for assay. Enrichment for CD4^+ cells was performed with the use of CD4 antibody conjugated to magnetic beads according to the manufacturer's protocol (Dynal, Inc.). The isolated cells were used for the extraction of total RNA.

Immunoprecipitation. 293 cells were transfected with combinations of expression plasmids: fl-IRF-7 expression plasmid alone; BZLF-1 alone; fl-IRF-7 and BZLF-1; fl-IRF-7 and LMP-1; and finally fl-IRF-7, LMP-1, and BZLF-1 (36). Cells were harvested after 48 h and lysed in immunoprecipitation lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 5 mM dithiothreitol, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and protease inhibitor mixture [Complete]). Cellular debris were removed by centrifugation at 10,000 rpm for 10 min, and the extracts were incubated with either 2 μg of Stratagene FLAG M2 antibody or 2 μg of BZLF-1 (Argene BZ-1) antibody for 1 h, and then 30 μl of protein A/G-Sepharose beads (Santa Cruz Biotechnology) was added, followed by incubation overnight with extracts at 4°C. Immune complexes were pelleted and washed four times with NP-40 lysis buffer. Beads were eluted by boiling the beads for 5 min in 1× sodium dodecyl sulfate (SDS) loading buffer. Eluted proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose, and immunoblotted with antibody to either IRF-7 or BZLF-1, and detection was performed with an enhanced chemiluminescence ECL kit (Amersham).

RT real-time PCR. Reverse transcription (RT) was carried out for 60 min in a volume of 20 μl with 1 μg of total RNA with the use of the RT-PCR system kit (Promega). Real-time PCR was performed with the ABI Prism 7900 sequence detection system (Perkin-Elmer, Foster City, CA). Tap-2 probes (Applied Biosystems and Assays by Design) were labeled at the 5' end with the reporter dye FAM and at the 3' end with the quencher dye TAMRA. The TaqMan GAPDH (glyceraldehyde-3-phosphate dehydrogenase) control probe was labeled at the 5' end with the reporter dye TET and at the 3' end with the quencher dye TAMRA. Tap-2 primer and probe sequences are as follows: sense primer, 5'-CGGCTCC CTGACCTGAGA-3'; antisense primer, 5'-CTGAAGCAGCCACAGTAAAG C-3'; probe 5'-CAGCAGGGAGGTCCAG-3'. PCR analyses of cDNA specimens and standards were conducted in triplicate in a total volume of 15 μl with

$2\times$ AmpliTaq Gold PCR Mastermix. Thermal cycler parameters were as follows: 2 min at 50°C, 2 min at 95°C, and 40 cycles involving denaturation at 95°C for 15 s and annealing and extension at 56°C for 1 min. The threshold was set in the linear region above baseline noise, and the threshold cycle (C_T) was determined as the cycle number at which the threshold line crossed the amplification curve. $\Delta\Delta C_T$ represented the GAPDH-normalized ratio between the empty vector control and the BZLF-1 reactivated sample. Measurements and calculations were made according to recommendations of the manufacturer (PE Applied Biosystems).

HLP and immunofluorescence. Tissue specimens of oral HLP were obtained at biopsy from human immunodeficiency virus (HIV)-seropositive patients identified at the University of North Carolina (UNC) Hospitals as described previously (83). Frozen HLP tissue sections were stained with primary antibody BZLF-1 (mouse monoclonal BZ1; Argene), LMP-1 (mouse monoclonal OT22C; Organon Teknika, Boxtel, The Netherlands), and IRF-7 (rabbit polyclonal; Santa Cruz); washed; stained with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (IgG) or lissamine rhodamine goat anti-rabbit IgG as described previously (83); and subjected to confocal microscopy. EGFP-IRF-7 293 cells were examined under inverted fluorescence microscopy; ca. 150 cells were counted for each sample.

RESULTS

IRF-7 is present in HLP lesions. Given that LMP-1 is expressed throughout HLP tissue and that in latently EBV-infected lymphocytes LMP-1 induces both expression of IRF-7 mRNA and activation of IRF-7 protein, we investigated whether IRF-7 is expressed in this cytolytic epithelial infection state.

HLP specimens subjected to fluorescence microscopy revealed typical ballooning degeneration around dense nuclei, pyknotic in appearance, that are characteristic of the EBV cytopathic effect in tongue epithelial cells (Fig. 1). Confocal fluorescence microscopy was used to detect and localize IRF-7, BZLF-1, and LMP-1 proteins in HLP lesions. Included as negative controls were sections stained with antibody to IRF-2 and a specimen of normal tongue from an HIV-seronegative patient.

Abundant BZLF-1 protein was detected in the nucleus of most cells in the middle to upper stratum spinosum (Fig. 1B), while characteristic membrane patching of LMP-1 was also noted in most cells of the middle to upper strata (Fig. 1E, mid-stratum shown). Interestingly, although IRF-7 is normally not expressed in cells of epithelial origin, and it is not detected in normal tongue specimens (Fig. 1I), IRF-7 was detected in abundance in the cells of the middle- to upper-stratum spinosum (Fig. 1A, middle to upper strata; Fig. 1D, mid-stratum) of the HLP lesion. These findings were localized to areas where cytopathic effects were evident in serial sections. Furthermore, IRF-7 was detected in both the cytoplasm and the nucleus of these infected keratinocytes (Fig. 1A and D). In contrast, IRF-2 was not detected (Fig. 1H).

Thus LMP-1 and BZLF-1 are readily detected in the stratum spinosum of HLP lesions. IRF-7 was detected in both the nucleus and the cytoplasm in the middle and upper strata of the HLP lesion in which LMP-1 and BZLF-1 are also expressed. These results indicate that IRF-7 is present in cells in which abundant viral cytolytic replication is occurring; its nuclear localization suggests that phosphorylation and translocation of the protein have occurred.

IRF-7 and BZLF-1 physically associate. Since both IRF-7 and BZLF-1 are detected in the nucleus of cells in HLP lesions and since BZLF-1 interacts with a number of cellular proteins,

we examined whether BZLF-1 and IRF-7 associated physically. 293 cells were transfected with combinations of expression plasmids: the Flag epitope-tagged IRF-7 expression plasmid alone, BZLF-1 alone, IRF-7 and BZLF-1, IRF-7 and LMP-1, and finally IRF-7, LMP-1, and BZLF-1. Cells were harvested after 48 h, and lysates were immunoprecipitated with antibody to the Flag epitope, subjected to SDS-PAGE, and immunoblotted with antibody to IRF-7 or to BZLF-1. As shown in Fig. 2A, BZLF-1 protein is coprecipitated when IRF-7 is expressed together with BZLF-1 and lysates are immunoprecipitated for IRF-7 but not when BZLF-1 is expressed alone. Also, after immunoprecipitation of these lysates with BZ1 antibody (Argene), IRF-7 protein again coprecipitated with BZLF-1. This interaction is independent of whether LMP-1 is present or not (Fig. 2B). To confirm whether endogenously expressed IRF-7 and BZLF-1 associate, we treated Raji cells, which express IRF-7, with methotrexate to induce the expression of BZLF-1 (17). After 48 h immunoprecipitation from Raji cells lysate brought down both BZLF-1 and IRF-7, whereas normal mouse IgG brought down neither, confirming that BZLF-1 and IRF-7 proteins at physiologic levels found in infected cells physically associate.

LMP-1-induced nuclear accumulation of IRF-7 is not inhibited by BZLF-1. Since BZLF-1 and IRF-7 can associate, and IRF-7 is detected in the nucleus of cells that also express abundant BZLF-1, we examined whether BZLF-1 affected the distribution of inactive or active IRF-7. The effect of BZLF-1 on IRF-7's nuclear accumulation was tested by transfecting EBV-negative 293 cells with expression vectors for EGFP-tagged IRF-7, BZLF-1, and LMP-1. Most of cells expressing enhanced green fluorescent protein (EGFP)-tagged IRF-7 fluorescence was detected only in the cytoplasm, whereas just 7% of cells showed fluorescence in the nucleus (Fig. 3A). However, coexpression of LMP-1 and EGFP–IRF-7 produced a marked shift in the distribution of EGFP–IRF-7. With IRF-7 now present in the nucleus of 66% of cells (Fig. 3B), IRF-7 distribution remained unchanged when EGFP–IRF-7 was coexpressed with BZLF-1 compared to when it is expressed alone (12% nuclear, Fig. 3C). In samples transfected with EGFP–IRF-7, LMP-1, and BZLF-1, no difference was observed compared to samples transfected with EGFP–IRF-7 and LMP-1 (72% versus 66%, Fig. 3B and D, both nuclear and cytoplasmic distribution). Thus, BZLF-1 does not seem to affect the subcellular distribution pattern of IRF-7, which is consistent with the distribution of IRF-7 in HLP epithelial cells (Fig. 1).

IFN promoter-reporter activity is inhibited during viral reactivation in EBV-positive B-cell lines. The presence of IRF-7 in the nucleus of HLP cells, which have abundant cytolytic EBV replication, suggests that IRF-7 is active and functional during viral replication. To determine whether IRF-7 was transcriptionally active during the lytic cycle, the promoter activity of IRF-7-responsive genes was assayed with the use of promoter-reporter constructs in latently infected cells during viral reactivation.

IFN-β and IFN-α4 are well-established targets of IRF-7. Their promoter activities are commonly used indicators of IRF-7 activity, as is an ISRE reporter-promoter construct (35, 38, 76, 85, 86). To assay IRF-7 activity during viral reactivation, the IFN-β promoter-reporter construct was transfected, along with an expression plasmid of BZLF-1, into X50-7 cells or Raji
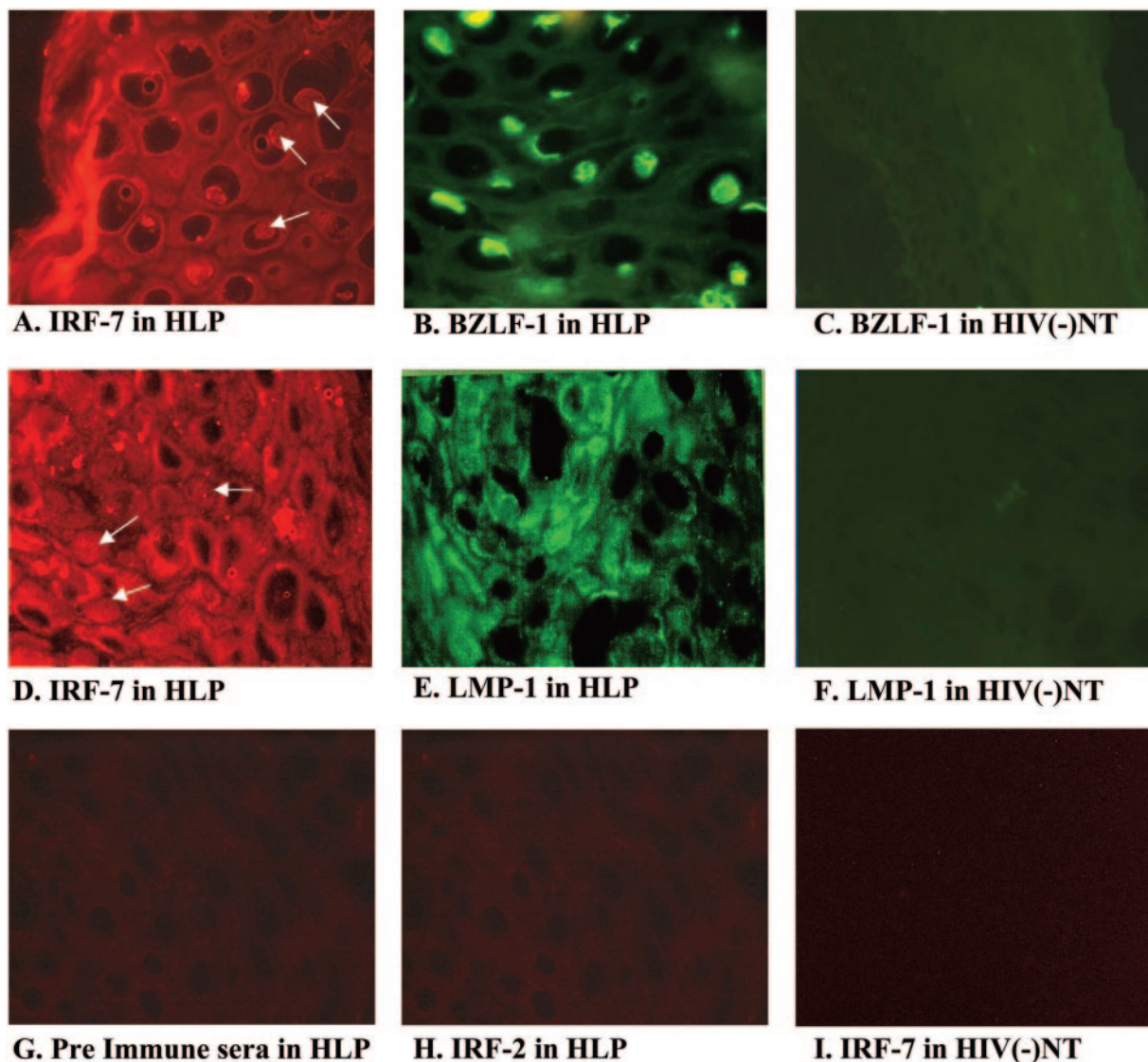
FIG. 1. IRF-7 is expressed in lesions of oral hairy leukoplakia and can be detected in cellular nuclei. Frozen serial sections of HLP tissue were stained with antibodies to the indicated proteins and subjected to confocal microscopy. (A) IRF-7 (red) is detected in the nucleus, as well as the cytoplasm, of cells in the middle and upper stratum spinosum of an HLP lesion (arrows indicate nuclei). (B) BZLF-1 (green) is detected in the nucleus of cells in a corresponding region of a serial section of the same HLP lesion. (C) BZLF-1 is not detected in normal tongue (NT). (D) IRF-7 (red) expression in both the nucleus and the cytoplasm of cells in the middle stratum spinosum. (E) LMP-1 (green) is detected on the cell surface of cells in the middle stratum spinosum. (F) LMP-1 is not detected in normal tongue. (G) Preimmune rabbit serum. (H) IRF-2 is not detected in HLP. (I) IRF-7 is not detected in normal tongue. Magnification, ×400.

cells to induce lytic reactivation. Control samples were cotransfected with the IFN-β construct and the vector control plasmid. After 24 h, induction of viral replication was assessed by immunoblotting for the appearance of the early gene, *EA-D* (data not shown). IFN-β promoter-reporter activity was measured by firefly luciferase assays and was normalized to *Renilla* luciferase expression.

The activity of the IFN-β reporter decreased by 60% in X50-7 cells in which lytic replication had been induced by expression of BZLF-1 protein compared to cells transfected with vector alone (Fig. 4A). In another type 3 cell line, Raji, IFN-β reporter activity decreased almost 80% in cells in which viral reactivation was induced.

The IFN-β promoter is responsive to other factors in addition to IRFs, such as NF-κB and ATF2/c-Jun (28). Therefore, a simplified reporter construct was used to limit involvement of other factors. The ISRE construct consists of five copies of the consensus IRF-binding site and is commonly used as an indicator of IRF activity (43, 70). This construct was cotransfected as before into X50-7 cells with either control vector or BZLF-1 expression plasmid. Relative ISRE reporter activity decreased by more than 60% in cells transfected with the BZLF-1 expression plasmid (Fig. 4B).

The ISRE construct interacts with a number of IRFs. IRFs display individual binding affinities and specificities for different ISRE sequences. IRF-3 for instance, possesses a restricted
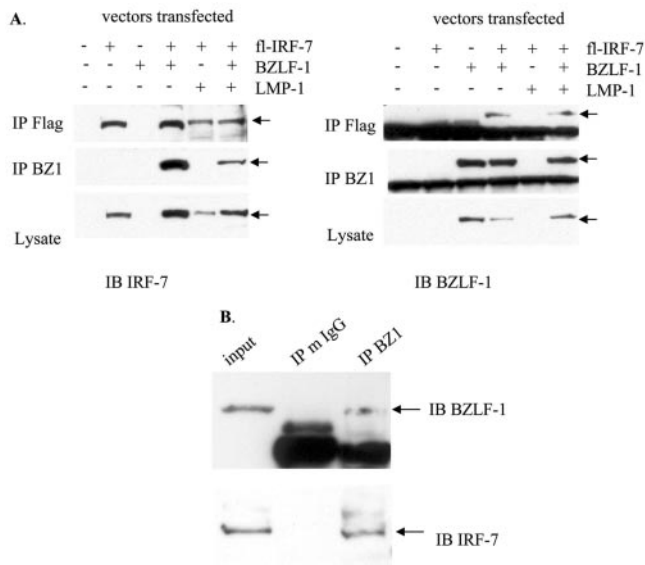
FIG. 2. IRF-7 and BZLF-1 proteins physically associate. (A) 293 cells were transfected with the indicated expression plasmids, and IRF-7 (FL-IRF-7) and BZLF-1 were immunoprecipitated with Flag antibody or BZ-1 antibody, respectively. Immunocomplexes were subjected to SDS-PAGE and immunoblotted for IRF-7 (arrows, left panel) or BZLF-1 (arrows, right panel). (B) Endogenous IRF-7 and BZLF-1 proteins physically associate in Raji cells in which virus replication has been induced. Raji cells were treated with 5 μg of methotrexate/ml for 48 h, BZLF-1 was immunoprecipitated from the cell lysates, and the immunocomplexes were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted for IRF-7 and BZLF-1. Arrows indicate IRF-7 (right panels) and BZLF-1 (left panels).

DNA-binding site specificity, and it only weakly binds the ISRE of the IFN-α4 promoter (GAAAATGGAAATT). This specificity prevents it from inducing IFN-α4 promoter activity (35, 86). IRF-7, on the other hand, has a broader range of

DNA-binding specificities [GAA(A/T)N(C/T)GAAANTC] than IRF-1, IRF-2, and IRF-3 [GAAA(C/G)(C/G)AAAN(T/C)] (35, 75, 86). This increased flexibility allows IRF-7 to bind to and activate the IFN-α4 promoter. For this reason IFN-α4 is used as a more precise indicator of IRF-7 activity (30). IFN-α4 promoter-reporter plasmid was cotransfected with either control vector or BZLF-1 expression plasmid and reporter activity measured as before. Transfection with BZLF-1 decreased relative IFN-α4 reporter activity by almost 60% (Fig. 4C).

These experiments demonstrate that the activities of IRF-responsive promoters are decreased during cytolytic EBV reactivation, whereas the activity of the internal control reporter pRL-TK, under the control of the thymidine kinase promoter, was not affected (data not shown). Specifically, the reduction in ISRE-mediated activity argues that IRF activity is reduced during lytic replication and, when considered together with a similar reduction in IFN-α4 promoter activity, suggests that a reduction in IRF-7 activity is likely to be responsible for decreased activities of these IRF-responsive promoters.

Tap-2 promoter activity is decreased during cytolytic reactivation. LMP-1 induces expression of the Tap-2 protein (60, 77, 91, 94) in an ISRE-dependent manner (91). Furthermore, in cells in which IRF-7 is not induced by LMP-1, Tap-2 is not induced by the viral protein (91). Since the Tap-2 protein is an essential component of the antigen presentation machinery of the MHC class I immune response pathway (2) and since MHC class I antigen presentation itself is reduced during viral replication (31), we tested whether the Tap-2 gene is downregulated during EBV reactivation.

To assay Tap-2 promoter activity during the cytolytic cycle, the Tap-2 promoter reporter was cotransfected with either the control vector or with the BZLF-1 expression plasmid, and CAT assays were performed on the lysates after 24 h. Comparison of these samples in Fig. 5A revealed a 70% reduction
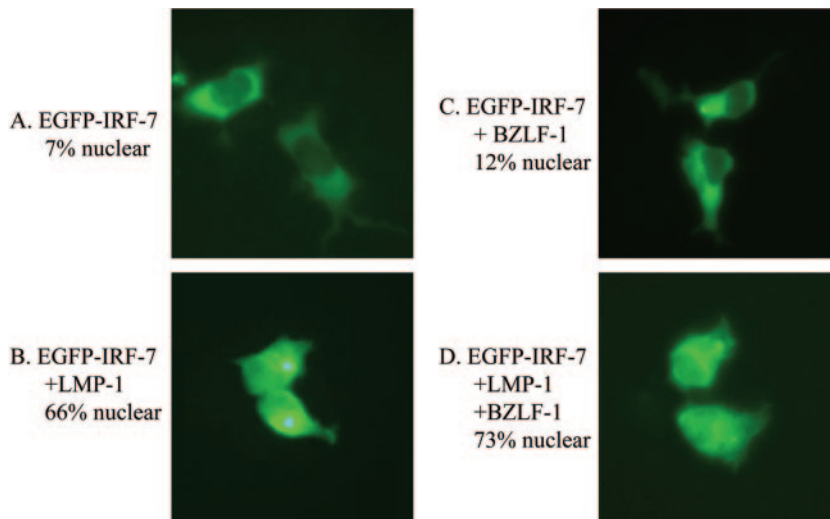

FIG. 3. Nuclear accumulation of GFP-IRF7 induced by LMP-1 is not inhibited by BZLF1. GFP fluorescence was detected by inverted light microscopy in 293 cells transfected with EGFP–IRF-7 (A); EGFP–IRF-7 and LMP-1 (B); EGFP-IRF-7 and BZLF-1 (C); or EGFP–IRF-7, LMP-1, and BZLF-1 (D). Subcellular localization of IRF-7 was examined 24 h after transfection. Fields of cells were scored to determine percentage of nuclear localization.
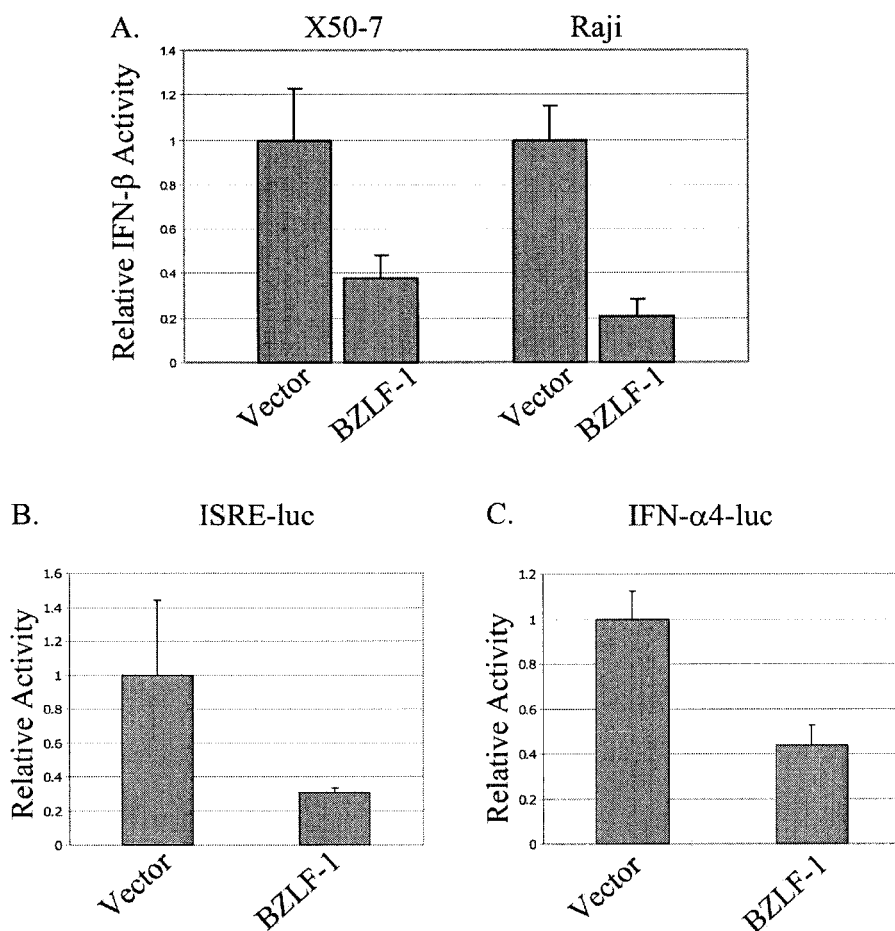
FIG. 4. IFN-β, IFN-α4, and ISRE promoter activity is reduced in EBV-positive cells in which cytolytic viral reactivation has been induced. X50-7 or Raji cells were transfected with 10 μg of reporter plasmid and either BZLF-1 expression vector or control vector. At 24 h after transfection, cells were harvested for firefly luciferase assay and normalized to *Renilla* luciferase expression. (A) IFN-β promoter-reporter activity in induced X50-7 and Raji cells. (B) ISRE reporter construct activity in induced X50-7 cells. (C) IFN-α4 reporter-promoter activity in induced X50-7 cells.

in Tap-2 promoter-reporter activity in cells in which lytic replication had been reactivated.

Mutation of the Tap-2 promoter ISRE from GA<u>AA</u>GCGA<u>AA</u>GC to GA<u>GGG</u>CGA<u>GGGG</u>C disrupts IRF interaction and activation (91). This mutated promoter construct (mTap-2 promoter) was used to determine whether the effect of Tap-2 promoter downregulation during lytic reactivation was ISRE dependent. Disruption of the ISRE reduced Tap-2 promoter activity by 60% compared to activity of the nonmutated Tap-2 promoter.

The activity of the mTap-2 promoter was only 40% that of the nonmutated promoter construct, and no further reduction of its activity was detected with reactivation of viral replication.

The reduced Tap-2 promoter-reporter activity was confirmed by quantitating relative levels of endogenous Tap-2 RNA. A portion of each of the X50-7 cells used above, all of which had been transfected with CD4 expression vector, were pooled and selected for the transfected cells by using magnetic beads conjugated to CD4 antibody (Dynal, Inc.). The vector control set and the BZLF-1 set of transfected cells were harvested, and the RNA was purified and subjected to RT real-

time PCR with primers to Tap-2 and GAPDH cDNA. Relative RNA levels were measured with the use of $\Delta\Delta C_T$ values. Endogenous Tap-2 RNA levels were reduced by 70% in X50-7 cells after induction of viral reactivation. Thus, viral reactivation resulted in a decrease in both TAP-2 RNA levels and promoter activity, and the decrease in promoter activity was ISRE dependent.

BZLF-1 inhibits IRF-7 activation by LMP-1. The findings in Fig. 4 and 5 demonstrate that IRF-7-responsive promoters are downregulated during EBV reactivation. However, they did not indicate whether a specific viral protein expressed during viral reactivation was responsible for this reduction, nor did they clearly demonstrate that it was the activity of IRF-7 on the responsive promoters that had been targeted. To measure IRF-7-dependent promoter activity, 293 cells were transfected with the IFN-β reporter and harvested, and the luciferase activity was analyzed. Expression of IRF-7 and LMP-1 separately had little effect on the IFN-β promoter (Fig. 6A). However, when coexpressed, a clearly synergistic activation is evident, i.e., >20-fold activation compared to IRF-7 expression alone.
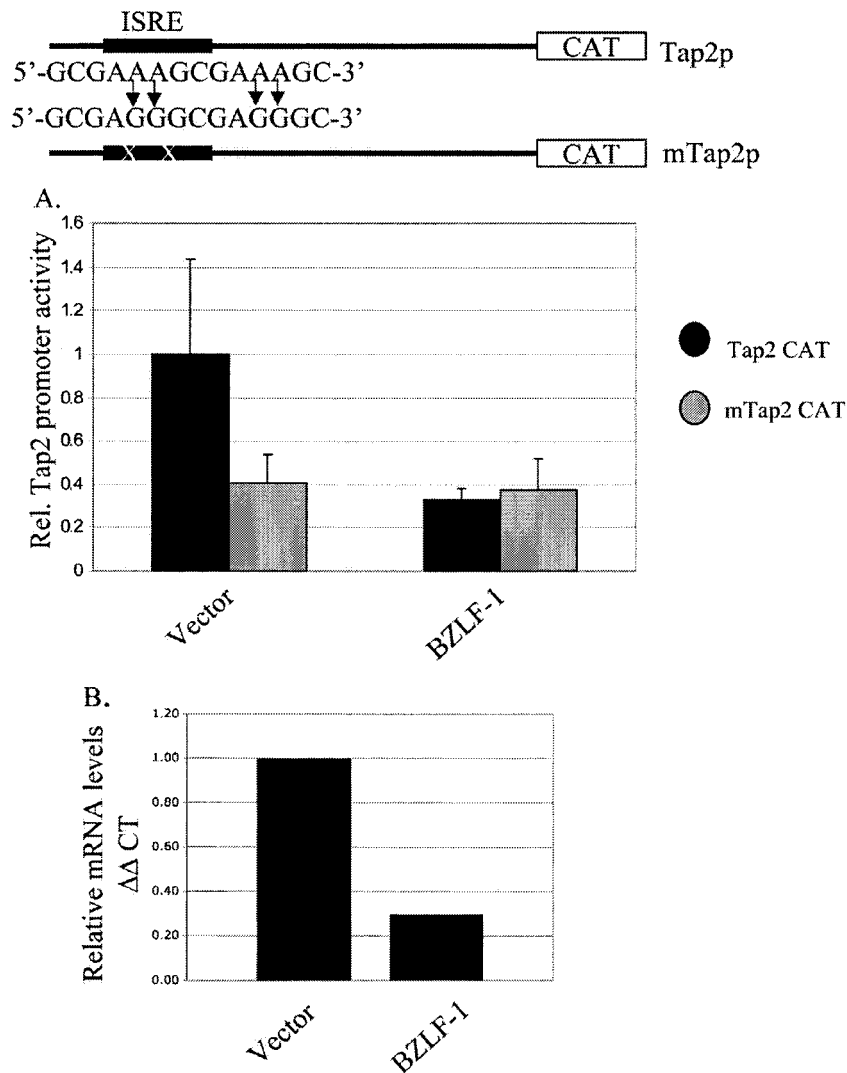
FIG. 5. TAP2 promoter activity is decreased in an ISRE-dependent manner in cells in which cytolytic replication has been induced. (A) X50-7 cells were nucleoporated (Amaxa) with either Tap-2 or mTap-2 promoter-reporter constructs, together with BZLF-1 or the vector control plasmids, and CD4 plasmid to a total of 5 μg of DNA. At 24 h after transfection, cells were harvested, a portion of which were used for CAT assays. The results were normalized with *Renilla* luciferase expression. Positions of point mutations are indicated at the top. (B) Endogenous TAP2 mRNA level is decreased during cytolytic viral reactivation. Remaining portions of the samples in panel A were pooled and enriched for transfected cells by CD4 selection. RNA was purified and used for real-time RT-PCR with Tap-2 mRNA specific probes. $\Delta\Delta C_T$ values represent the GAPDH-normalized ratio between the empty vector control and the BZLF-1-induced sample.

With this system EBV lytic proteins could then be tested for their effect on IRF-7 activity in the absence of other viral factors or lytic replication. In the course of the experiments described above, two EBV lytic gene products, BKRF-4 and BZLF-1 itself, were selected as possible candidates that might inhibit IRF-7 activity. BZLF-1 was tested because it interacts with and modulates a number of cellular proteins involved in cellular and immune responses. In addition, BZLF-1 induces viral reactivation in type 3 latently infected cells, and so it was necessary to control for any effects the protein itself might have on IRF-7 activity. BKRF-4 was evaluated because it is the homolog of KSHV ORF45, which has been reported to inhibit IRF-7 activity. *BKRF-4* is a late gene, the RNA of which is expressed beginning ~6 h after induction of viral replication (A. M. Hahn and J. S. Pagano, unpublished observations).

As shown in Fig. 6A, in EBV-negative cells expression of BZLF-1 reduced the activity of IRF-7 on the IFN-β promoter by almost 70%. Furthermore, IRF-7 activation by LMP-1, as shown by reporter assay in Fig. 6A, is inhibited by BZLF-1 in a dose-dependent manner. These results were confirmed in HeLa and C33A cell lines (data not shown). BKRF-4 was also coexpressed with LMP-1 and IRF-7 in this reporter assay. In contrast to BZLF-1, BKRF-4 had no detectable effect in this assay of IRF-7 activity, a finding which suggests that BKRF-4 may not have the same inhibitory activity on IRF-7 as reported for its KSHV homolog, as well as indicates that inhibition of IRF-7 activity is not a general effect of expression of a lytic cycle viral protein.

The IFN-β promoter is responsive not only to IRFs but also to NF-κB and ATF/c-Jun. To limit the effect of these factors,
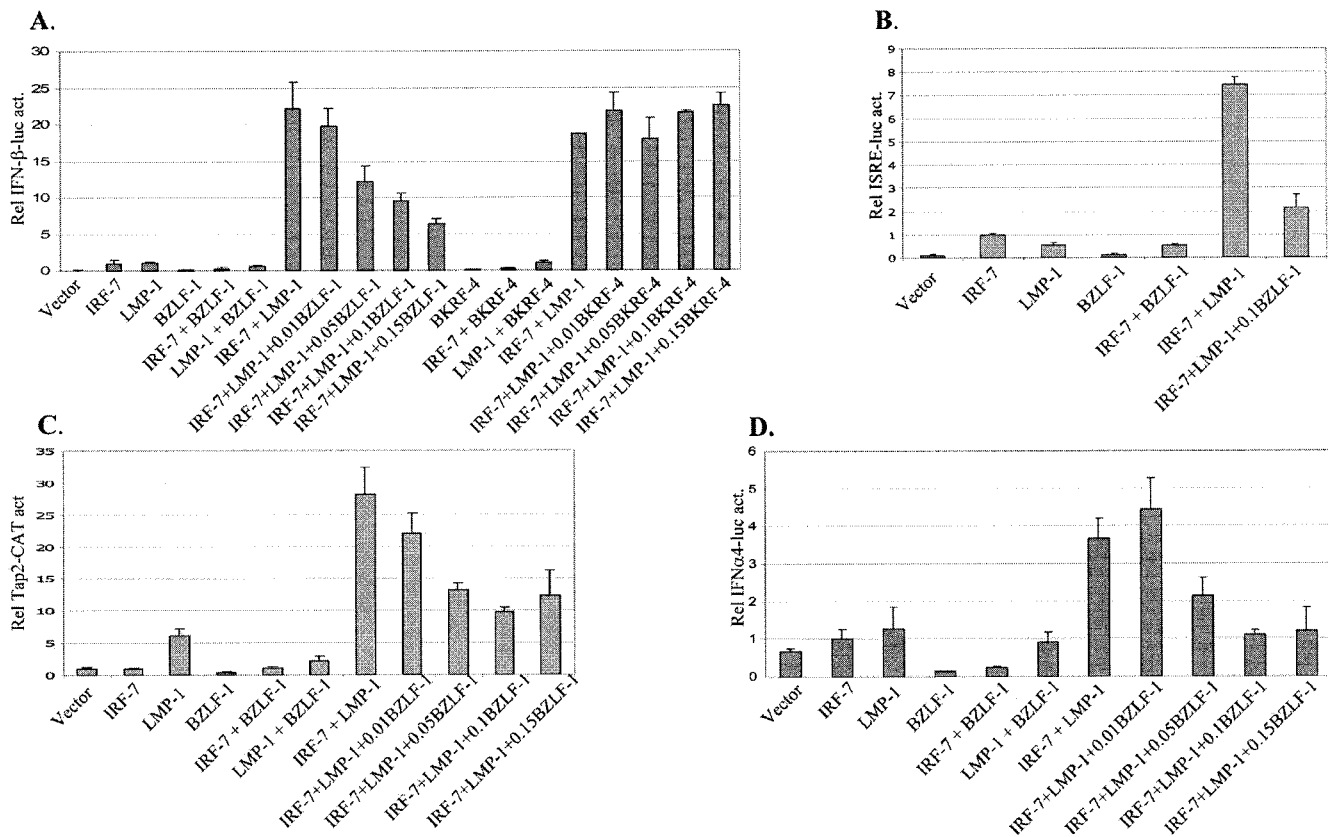
FIG. 6. BZLF1 modulates the activation of IRF-7 by LMP1 in a dose dependent manner. 293 cells in 12-well plates were transfected with reporter plasmids (0.1 μg of IFN-β-luc, IFN-α4-luc, Tap-2 CAT, and ISRE-luc; 0.025 μg of pRL-TK) and expression plasmids encoding IRF-7 (0.1 μg), LMP-1 (0.1 μg), BZLF-1 (amounts as indicated), and BKRF-4 (amounts as indicated). Luciferase (A, B, and D) or CAT (C) assays were performed, and data were normalized to *Renilla* luciferase. Values are shown as the fold activation relative to IRF-7. (A) BZLF-1 inhibits LMP-1-induced activation of the IFN-β-luc promoter reporter by IRF-7. (B) BZLF-1 inhibits LMP-1-induced activation of the ISRE-luc reporter by IRF-7. (C) BZLF-1 inhibits LMP-1-induced activation of the Tap-2–CAT promoter reporter by IRF-7. (D) BZLF-1 inhibits LMP-1-induced activation of the IFN-α4-luc reporter by IRF7.

the ISRE reporter construct was used to measure IRF-7 activity. In the reporter assay results depicted in Fig. 6B, IRF-7 and LMP-1 had little effect separately but together produced sevenfold higher activity in this construct than IRF-7 expression by itself. Expression of BZLF-1 plasmid reduced this activity by 70% (Fig. 6B).

The Tap-2 promoter exhibits ISRE-dependent LMP-1 activation. As shown in Fig. 5A, the activity of the Tap-2 promoter is reduced during viral reactivation in X50-7 cells. To determine whether this reduction is due to the negative regulation of IRF-7 by BZLF-1 in the absence of viral replication, cells were cotransfected with the Tap-2 promoter-reporter construct along with either IRF-7, LMP-1, or IRF-7 and LMP-1. As shown in Fig. 6C, IRF-7 alone had little effect on this promoter, but when expressed with LMP-1 Tap-2 promoter activity was increased more than 25-fold. Expression of BZLF-1 plasmid reduced this activity by 60%. Thus, BZLF-1 can negatively regulate the activation of the Tap-2 promoter by the LMP-1/IRF-7 pathway.

Due to IRF-7's relaxed DNA-binding specificity compared to other IRF family members, IRF-7 is able to activate the IFN-α4 promoter. The IFN-α4 reporter was therefore used as

a more precise indicator of IRF-7 activity (30). As shown in Fig. 6D, IRF-7 is activated by LMP-1 as indicated by a 3.5-fold increase in IFN-α4 promoter activity, and BZLF-1 inhibited this activity by more than 60%.

Since the effects of LMP-1 and BZLF-1 on IFN-β promoter activity might be due to effects on IFN signaling rather than directly on IRF-7 activity, we next used the U4A cell line, which is deficient for Jak1 expression (41). IFN signaling is dependent on Jak1, and this cell line makes it possible to assay IRF-7 activity in the absence of IFN feedback. In data not shown, the expression of either IRF-7 or LMP-1 results in negligible activity as assayed with the ISRE reporter construct, but together they activate the reporter sevenfold. This activity can be reduced almost 80% by BZLF-1 protein in the U4A cells (data not shown). The parental 2fTHG cell line, in which Jak1 is intact, shows similar results. Expression of IRF-7 or LMP-1 separately results in little activity of the ISRE construct, but together they activate the reporter 5.5-fold. This activity can be reduced by almost 65% by BZLF-1 protein. These results indicated that IFN has no role in either the activation of IRF-7 by LMP-1 or in inhibition of that activity by BZLF-1.
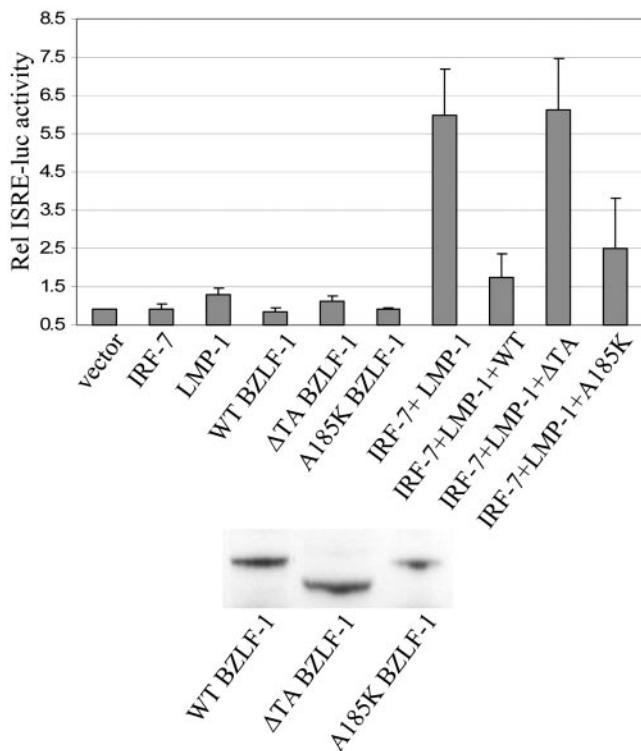
FIG. 7. Deletion of the transactivation domain of BZLF1 inhibits its ability to deactivate IRF-7. 293 cells were transfected with reporter plasmids and expression plasmids encoding IRF-7, LMP-1, BZLF-1, BZLF-1 ΔTA, and BZLF-1 A185K as indicated. Luciferase assays were performed, and data were normalized to *Renilla* luciferase. Values are shown as the fold activation relative to IRF-7. Lower panel shows immunoblot for WT BZLF-1, BZLF-1 ΔTA, and BZLF-1 A185K.

Deletion of the activation domain of BZLF-1 reduces its ability to inhibit IRF-7, whereas the DNA-binding domain is not required. To determine which domains of BZLF-1 are required for the inhibition of IRF-7 activity, mutants of BZLF-1 (50) with either a deletion of its activation domain or mutation of the DNA-binding domain were cotransfected into 293 cells with the ISRE reporter construct, together with IRF-7, LMP-1, or both. When LMP-1 is expressed, IRF-7 has a sixfold-higher activity (Fig. 7), but when cotransfected with wild-type BZLF-1 this activity was reduced 70%. Deletion of the activation domain of BZLF-1 abolished its effect on LMP-1-dependent IRF-7 activation, whereas mutation of the BZLF-1 DNA-binding domain was as effective as wild-type BZLF-1 in inhibiting IRF-7 activity (Fig. 7). Thus, the ability of BZLF-1 to bind to DNA is not necessary for its effect on IRF-7 activity, whereas the activation domain of BZLF-1 is required.

IRF-7 activation through the dsRNA/TLR3 pathway is inhibited by BZLF-1. Since BZLF-1 may inhibit the LMP-1 activation pathway of IRF-7 rather than the activity of IRF-7, we wanted to activate IRF-7 by another mechanism. Toll-like receptor 3 (TLR-3) is the receptor through which dsRNA binds, signals, and activates IRF-7 (30). Stably expressing TLR-3 293 cells were transfected with the ISRE reporter construct and assayed for luciferase activity (Fig. 8A). Treatment of these cells with poly(I-C), a synthetic dsRNA, for 8 h produced a

sixfold increase in ISRE activity. Transfection of these cells with IRF-7 results in only minimal activity; however, treatment with poly(I-C) results in 14-fold activation of the ISRE reporter. BZLF-1 reduced dsRNA-mediated IRF-7 induced reporter activity by 40%. The results were comparable when IRF-7 was activated by Sendai virus (data not shown), thus showing that BZLF-1 can inhibit multiple IRF-7 activating pathways, specifically LMP-1, dsRNA/TLR-3, and viral activation of IRF-7.

Activation of the ISRE promoter by a constitutively active IRF-7 mutant is blocked by BZLF-1. Based on results showing that multiple IRF-7 activating pathways are inhibited by BZLF-1 (Fig. 6 and 8A and data not shown), whether BZLF-1's effect was independent of the pathway for activation of IRF-7 was tested with a constitutively active phosphomimetic mutant of IRF-7 (IRF-7D477/479). Substitution of Ser-477 and Ser-479 with aspartic acid residues mimics phosphorylation resulting from viral infection (36). The relative activities of wild-type IRF-7 and IRF-7D477/479 are compared in Fig. 8B. The constitutively active form is six times more active on the ISRE reporter construct than is wild-type IRF-7. BZLF-1 reduces the activity of IRF-7D477/479 by ca. 60%. Thus, BZLF-1 can inhibit functions of previously activated IRF-7 protein, and this, together with the results shown in Fig. 8A, indicates that BZLF-1 modulates IRF-7 not by preventing its activation through signaling but rather at the level of IRF-7 transactivity.

DISCUSSION

IRFs are increasingly recognized as broadly active transcription factors in many aspects of cell biology, including regulation of host defense and immune responses, cytokine signaling, regulation of cell growth, and hematopoietic development. IRF-7, which was first cloned as a transcriptional regulator of the central EBV latency gene, *EBNA-1*, is intricately associated with EBV infection (57, 88–92). In this study we extend this association from latent EBV infection to appreciation of the involvement of IRF-7 in cytolytic infection, in which quite different outcomes are detected.

We have shown that during latent infection expression of IRF-7 and LMP-1 appear to be reciprocally regulated. LMP-1 can induce and activate IRF-7, resulting in its phosphorylation and nuclear translocation. In addition, the promoter of LMP-1 contains an ISRE that responds to activation by IRF-7 (57). We have postulated that this feedback circuitry is involved in the oncogenic potential of EBV LMP-1 (58, 58a, 93). Here we demonstrate that IRF-7 is abundantly expressed in cytolytic EBV infection in vivo and that the immediate-early gene *BZLF-1* inhibits IRF-7 activation of several key IFN-responsive promoters. These findings are significant not only because they address how the virus handles the continuing expression of IRF-7 during lytic reactivation but also because they suggest how the virus may avoid the deleterious effects of the antiviral response during lytic infection. Furthermore, the finding that IRF-7 is expressed in vivo in HLP of the tongue, the quintessential example of cytolytic EBV infection in which replication of the virus dominates, indicates that the virus has triggered early stages of the antiviral response but that the resulting effects of that response may be muted, as indicated by the lack of inflammatory infiltrate.
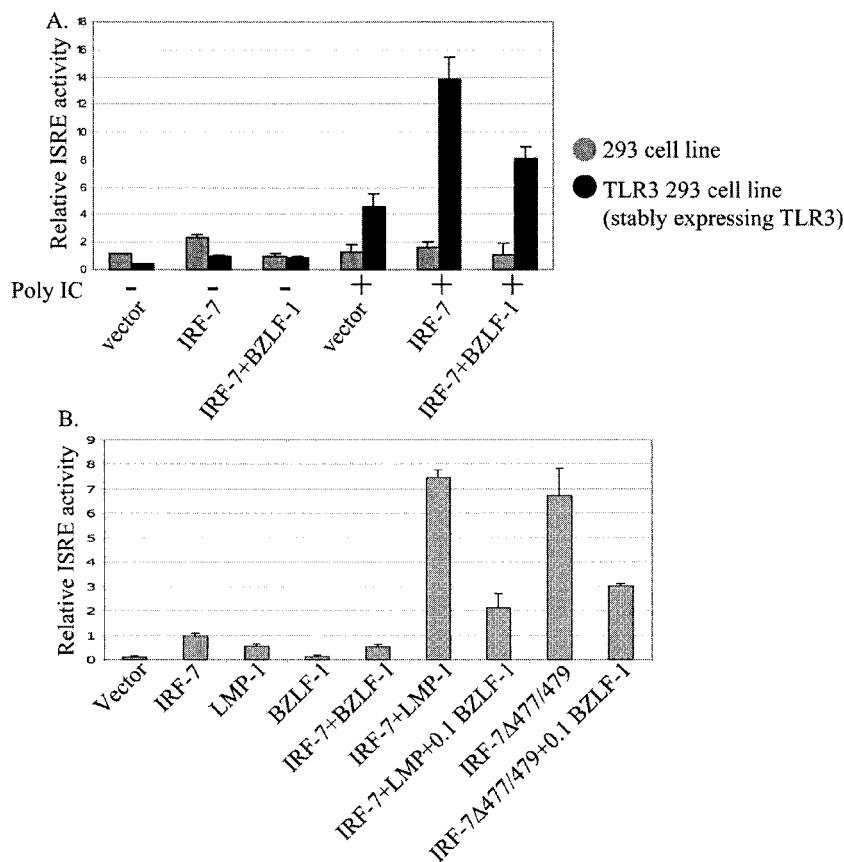
FIG. 8. Modulation of IRF7 by BZLF1 is downstream of signaling. (A) IRF-7 activation through the TLR-3/dsRNA pathway is inhibited by BZLF1. 293 cells and 293 cells stably expressing TLR-3 were transfected with reporter plasmids and expression plasmids for IRF-7, LMP-1, and BZLF-1 as indicated. Luciferase assays were performed, and data were normalized with *Renilla* luciferase. Values are shown as the fold activation relative to IRF-7. (B) Activation of the ISRE promoter by a constitutively active IRF7 mutant is blocked by BZLF-1. 293 cells were transfected with reporter plasmids and expression plasmids encoding IRF-7, IRF7 (D477/479), LMP-1, and BZLF-1 as indicated. Luciferase assays were performed as in panel A.

IRF-7 is detected in both the cytoplasm and the nucleus of cells in the HLP lesion. Nuclear accumulation of IRF-7, a hallmark of its activation, occurs after phosphorylation that results from induction by virus infection and by LMP-1 (91). The ubiquitous presence of LMP-1 in HLP sections, the ongoing lytic replication, and the nuclear accumulation of IRF-7 all suggest that IRF-7 has been activated in this lesion. Interestingly, BZLF-1 is also expressed in these cells, which provides an in vivo example of an EBV lytic infection in which both this viral protein and IRF-7 are expressed. Consistent with the nuclear detection of both IRF-7 and BZLF-1 in HLP, we demonstrated that in 293 cells, BZLF-1 had no effect on accumulation of IRF-7 in cells coexpressing LMP-1.

In the EBV-positive B-cell lines X50-7 and Raji lytic reactivation was induced by overexpressing BZLF-1. The resulting inhibition of transcriptional targets of IRF-7, IFN-α4, IFN-β, and Tap-2 suggests that one or more lytic cycle viral genes may be responsible for this downregulation. In 293 cells, which are EBV negative, BZLF-1 consistently negatively regulated IRF-7 activity in the absence of other viral factors and in the absence of viral replication. Thus, expression of BZLF-1 is sufficient to account for the inhibition of IRF-7-responsive promoters seen in induced X50-7 and Raji cells. Together with

our findings in HLP lesions, these observations may help to explain why, despite the presence of IRF-7, viral replication proceeds.

In examining the direct effects of BZLF-1, we found that the activation domain, but not the DNA-binding domain, of the protein is required for downregulation of IRF-7 activity. Also, that BZLF-1 can inhibit the activity of the phosphomimetic mutant of IRF-7 shows that BZLF-1 can negatively regulate IRF-7 even when already activated. Moreover, the fact that endogenous as well as overexpressed IRF-7 and BZLF-1 proteins physically associate suggests that protein-protein interactions may be part of the mechanism that BZLF-1 engages to inhibit IRF-7 activity.

Given that activated IRF-7 and BZLF-1 are nuclear proteins that physically associate, a possible model for this negative regulation is that IRF-7 is activated during EBV replication by LMP-1, is phosphorylated, and translocates to the nucleus. Once in the nucleus IRF-7 encounters BZLF-1, with which it interacts, resulting in dampening of IRF-7's transactivating ability. Mechanistically, BZLF-1 may then negatively regulate IRF-7 by either preventing it from binding to the ISRE of target promoters or by forming a complex with IRF-7 that binds DNA but can no longer function as a transactivator.

Several members of the herpesvirus family inhibit cellular antiviral responses through the production of immediate-early gene products. For instance, infection of cells with KSHV results in a massive increase in IRF7 protein levels, but corresponding microarray results fail to show upregulation of downstream IFN-αs or IFN-β mRNA in the infected cells (65). This effect is apparently due to the KSHV immediate-early product Rta, which blocks IRF-7-mediated IFN production and promotes IRF-7 ubiquitination and degradation (87). The mechanisms involved with the two viruses appear to differ. We did not specifically test the effect of BZLF-1 on IRF-7 protein stability; however, coexpression of BZLF-1 did not affect IRF-7 levels, as detected by immunoblotting or EGFP fluorescence (Fig. 2 and 3 and unpublished data).

KSHV also encodes a second immediate-early gene, *ORF45*, that was reported to inhibit virus-mediated activation of IRF-7 by interacting with IRF-7 in the cytoplasm, blocking its phosphorylation and consequently its nuclear accumulation. In contrast, neither BKRF-4, the EBV homolog of ORF45, nor BZLF-1 blocked translocation of IRF-7 to the nucleus (unpublished data and Fig. 3). We also tested BKRF-4 for the ability to downregulate the LMP-1-mediated IRF-7 activity of the IFN-β promoter. BKRF-4 and ORF45 are purported homologs due to sequence homology in both the amino and the carboxyl termini of the proteins (Hahn and Pagano, unpublished). We did not detect an effect of BKRF-4 on IRF-7 activity in this assay (Fig. 6A). Several reasons may account for this result: IRF-7 may not be a target of BKRF-4, BKRF-4 may not affect the LMP-1 signaling pathway which activates IRF-7, or BKRF-4 may require a cofactor expressed during lytic reactivation to downregulate IRF-7 activity.

In addition, HSV-1 infection of human fibroblasts in the absence of de novo protein synthesis could induce the expression of IFN-responsive genes. If viral replication was permitted induction of these genes was inhibited, raising the possibility that newly made viral products might function as inhibitors of this response (52, 53, 56, 66). Although the mechanism is not clear, HSV-1 ICP-0 negatively regulates IRF-3 and IRF-7 activity and inhibits the host antiviral response (15, 27, 37, 54). Lastly, human cytomegalovirus infection of human fibroblasts initiates but then blocks the induction of IFN-responsive genes (11–13, 44–46, 96, 97), perhaps through the virion protein, UL83, which inhibits the antiviral pathways, leading to activation of IRF-3, IRF-1, and NF-κB (1, 11).

The nuclear accumulation of IRF7 in HLP (Fig. 1A) suggests that IRF-7 has been activated. This is interesting because an important pathological characteristic of HLP lesions is that despite high levels of viral replication this tissue shows a reduction in the numbers of Langerhans cells and T lymphocytes, the opposite of what is expected in an infection in which IRF-7 protein has been induced and activated (14). Reductions in Langerhans cell counts through an unknown mechanism have been directly linked to EBV replication (80). Our findings suggest that BZLF-1 may play such a role by inhibiting the production of IFN-α/β, which is involved in activation, maturation, and migration of dendritic and Langerhans cells into infected tissue (39). Other EBV factors, such as viral interleukin-10 (vIL-10) and the gp25-gp42-gp85 glycoprotein complex, have been implicated (25, 34, 80). vIL-10 downregulates proinflammatory cytokines that are involved in the differentiation of CD14$^+$ monocytes into Langerhans cells (5, 6, 29, 48). The EBV glycoprotein complex inhibits dendritic cell differentiation and promotes apoptosis in CD14$^+$ monocytes (25, 34). All three viral factors may act in concert to prevent the inflammatory response in HLP: BZLF-1 inhibits the IFN response, which in turn prevents the activation and migration of Langerhans cells and T cells into HLP, whereas vIL-10 and the glycoprotein complex prevent the differentiation of preexisting monocytes into Langerhans cells.

Study of EBV-infected cells that had spontaneously entered the lytic cycle showed a significant decrease in MHC class I expression compared to latently infected cells (31). Downregulation of MHC class I expression occurred when the late viral lytic cycle was blocked by treatment with acyclovir, which suggests that the effect was due to an immediate-early or early gene (31). Further, BZLF-1 inhibited the upregulation of MHC class I expression mediated by EBV LMP1 (31). Our results demonstrate that BZLF-1, in the context of viral replication, inhibits activation of the Tap-2 promoter, an essential component of MHC class I antigen presentation. Inhibition of the Tap-2 promoter is ISRE dependent, and BZLF-1 can directly inhibit the LMP-1-mediated activation of IRF-7 of the Tap-2 promoter. It is possible that antigen presentation is inhibited by BZLF-1 through the downregulation of this principal gene involved in peptide transport as well as other MHC molecules. Such a mechanism might aid in establishing a state of immunologic ignorance during viral replication (31).

In summary, we have demonstrated that BZLF-1 interacts with and inhibits IRF-7 activity on the IFN-α4, IFN-β, and Tap-2 promoters. This negative regulation of IRF-7 activity may provide a mechanism by which the virus evades cellular antiviral responses and immune detection. Moreover, the inhibition of IRF-7 activity by BZLF-1 may help explain the lack of inflammatory infiltrate in HLP lesions, as well as the reduction in MHC class I expression in lytic replication. Thus, BZLF-1 is able to inhibit IRF-7, one of the most critical factors in innate and adaptive immune responses to viral infection.

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