The bZIP Transactivator of Epstein-Barr Virus, BZLF1, Functionally and Physically Interacts with the p65 Subunit of NF- κ B

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Received 20 May 1993/Returned for modification 9 July 1993/Accepted 13 December 1993

The Epstein-Barr virus (EBV) BZLF1 (Z) immediate-early transactivator initiates the switch between latent and productive infection in B cells. The Z protein, which has homology to the basic leucine zipper protein c-Fos, transactivates the promoters of several replicative cycle proteins. Transactivation efficiency of the EBV BMRF1 promoter by Z is cell type dependent. In B cells, in which EBV typically exists in a latent form, Z activates the BMRF1 promoter inefficiently. We have discovered that the p65 component of the cellular factor NF- κ B inhibits transactivation of several EBV promoters by Z. Furthermore, the inhibitor of NF- κ B, I κ B α , can augment Z-induced transactivation in the B-cell line Raji. Using glutathione S-transferase fusion proteins and coimmunoprecipitation studies, we demonstrate a direct interaction between Z and p65. This physical interaction, which requires the dimerization domain of Z and the Rel homology domain of p65, can be demonstrated both in vitro and in vivo. Inhibition of Z transactivation function by NF- κ B p65, or possibly by other Rel family proteins, may contribute to the inefficiency of Z transactivator function in B cells and may be a mechanism of maintaining B-cell-specific viral latency.

Epstein-Barr virus (EBV) is a human herpesvirus which infects primarily B cells and epithelial cells (reviewed in reference 33). Infection with EBV is strongly associated with the development of Burkitt's lymphoma in B cells and nasopharyngeal carcinoma (45) in epithelial cells. Infection of normal B cells with EBV results in the establishment of a latent state, with entry into the viral replicative cycle typically occurring in only a very small number of these cells (33, 45). In contrast, infection of epithelial cells with EBV results in viral production and shedding into the saliva (66). The BZLF1 (Z) protein is the first productive cycle protein that is expressed during reactivation of viral replication in B cells (14, 15, 70). The immediate-early Z protein disrupts viral latency through transactivation of early EBV genes which contain upstream Z-binding sites (9, 11, 13, 14, 16, 30, 31, 42, 43, 64, 71). Z also can activate its own expression (19, 71). Although the Z protein lacks the classic heptad leucine repeats, Z belongs to the basic leucine zipper (bZIP) family of proteins, containing a basic DNA-binding domain adjacent to a coiled-coil dimerization domain (18). Z protein structure bears resemblance to the structure of the c-Fos leucine zipper protein (18); however, unlike c-Fos, Z has not been shown to heterodimerize with any other bZIP protein.

We have shown that some EBV early promoters, such as the BMRF1 (early antigen-diffuse) promoter, are transactivated very poorly by Z in certain lymphoid cell lines, while in epithelial cell lines, the same promoters are highly Z responsive (29, 53). In lymphoid cell lines not supporting efficient Z function, there may exist inhibitors of Z transactivation. One candidate for such a B-cell-specific inhibitor of Z is the cellular factor NF- κ B.

As with Z, constitutive NF- κ B function is cell type specific (39, 63). Although NF- κ B is expressed in many types of cells, in most instances NF- κ B is complexed with I κ B and held in an inactive state within the cytoplasm (3). In mature B cells, in which NF- κ B function regulates the expression of the immunoglobulin kappa protein (39, 63), a portion of NF- κ B is dissociated from I κ B and thus found in the nucleus in an active form (3).

Classic NF- κ B is composed of two proteins, p50 and p65 (4). The p50-p65 complex can bind to NF- κ B sites within certain promoters (63) and activate transcription. These two proteins have been found to have structural similarity to the protein product of the *c-rel* proto-oncogene and other Rel-like proteins (21, 34, 52, 57). The p50 protein is processed from a larger precursor protein, p105, which contains a series of domains called ankyrin repeats (21, 34), and is also closely related to another ankyrin-containing Rel type protein called p100/p52 (50). The MAD-3 form of I κ B (I κ B α) contains ankyrin repeats without the Rel homology domain (27). A second group of Rel-like proteins, including p65 (52, 57), c-Rel (73), RelB (59), and the *Drosophila* protein Dorsal (69), all have Rel domains without ankyrin repeats. A newly discovered alternatively spliced form of p65, termed p65 Δ , lacks 10 amino acids within the Rel domain of p65 (58). Overexpression of this naturally occurring variant of p65 in Rat-1 cells produces transformed foci which are tumorigenic in nude mice (48). The complex patterns of heterodimerization between the Rel family members are now being elucidated (reviewed in reference 51). In addition to multimerization between Rel family proteins, interfamily interactions involving Rel proteins exist. Recently, it was found that the p50 and p65 proteins can physically interact with the bZIP protein, NF-IL6 (38, 67).

Here we report the physical and functional interaction between a member of the bZIP family of transactivators, Z, and the Rel protein, NF- κ B p65. We show that p65 functions

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as an inhibitor of Z-induced transactivation of early EBV promoters and that Z and p65 can physically interact with one another both in vitro and in vivo. This ability of p65 to inhibit the function of the EBV protein which triggers disruption of viral latency may partly explain the tendency of EBV infection to remain latent in B cells.

MATERIALS AND METHODS

Plasmid constructions. The pEA-BS-CAT construct, which contains the EBV BMRF1 promoter sequences from -331 to +1 linked to the chloramphenicol acetyltransferase (CAT) reporter, has been described elsewhere (32). The pBHRF1-CAT plasmid contains a 1,020-bp *NaeI*-to-*HincII* fragment of the EBV BHRF1 promoter (26) (EBV positions 52800 to 53819) linked to CAT in the plasmid vector pBS+ (Stratagene). The pHD1013 expression plasmid contains a 2.1-kb *PstI* fragment of the cytomegalovirus (CMV) immediate-early promoter/enhancer in the *PstI* site of pGEM2 (Promega) (30). Plasmid CMV-Z contains the Z cDNA from plasmid pKSVZ41 (a gift from Alain Sergeant) (22) downstream and under the control of the CMV immediate-early promoter in pHD1013. Construction of the CMV promoter control vector, pCMV4T, has been described elsewhere (72). Plasmids pCMV4T-p65, pCMV4T-p65Δ, and pCMV4T-IκB contain cDNAs encoding human NF-κB p65 (57), p65Δ (58), and IκBα/MAD-3 (27) inserted into the pCMV4T expression vector (67). Plasmid pGST-Z was created by inserting the cDNA encoding full-length Z (a gift of Paul Farrell) blunt end and in frame into the *SmaI* site of pGEX-3X (Pharmacia). Glutathione S-transferase (GST) ZΔ1-79, GST ZΔ1-133, and GST ZΔ200-227 contain Z cDNA lacking coding sequence for the amino acid residues indicated by the numbers used to name each plasmid (22, 32) (gifts of Alain Sergeant and Frank Furnari) inserted into pGEX-2T (Pharmacia). The GST-p65 construct contains the intact p65 coding sequences downstream of the GST protein (67) in plasmid pGEX-1N (AMRAD Corp.). The Z-containing *EcoRI* fragment from pKSVZ41 was inserted into a modified pFlag (International Biotechnologies, Inc.) vector (8) to produce pFlag Z. Similarly, a fragment encoding the first 171 amino acids of a Z cDNA was generated by PCR and inserted into the modified pFlag vector, creating pFlag ZΔCOOH. The plasmid for in vitro expression of the wild-type Z protein (a gift of Paul Farrell) and the point mutants of the Z dimerization domain (a gift of Erik Flemington and Samuel Speck) have been described in detail elsewhere (20, 56). Plasmids for in vitro expression of wild-type p65, p65Δ, and p65 with a four-amino-acid substitution from KRKR to DQNG in the nuclear localization signal of p65 (p65 ΔNLS; a gift of Amer Beg) have been described elsewhere (6, 67). The in vitro-translated proteins p65 Δ313, p65 Δ285, and p65 Δ196 were created by runoff transcription ending at *BspHI*, *EcoRI*, and *BglII* sites, respectively, within the wild-type p65 reading frame.

DNA preparation and transfections. Plasmid DNA was purified through two sequential cesium chloride gradients. Electroporation of both lymphoid and epithelial cells was performed as previously described (31, 70). Approximately 5×10^6 cells were used for each condition.

Cell lines. P3HR1 and Raji are EBV-positive Burkitt's lymphoma B-cell lines which were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. The cervical carcinoma cell line HeLa was grown in Dulbecco modified Eagle medium H with 5% fetal calf serum.

CAT assays. Cells were harvested 48 h after transfection with plasmid DNA. Cell extracts were incubated at 37°C with

^{14}C -labeled chloramphenicol in the presence of acetyl coenzyme A as previously described (24). The percent acetylation of chloramphenicol was quantitated by thin-layer chromatography, followed by autoradiography and either scintillation or PhosphorImager (Molecular Dynamics) counting. The CAT assays were repeated with less extract if results were not in the linear range (less than 70% acetylated).

Synthesis and radiolabeling of proteins. Plasmids encoding the GST fusion proteins were introduced into *Escherichia coli* HB101. An overnight culture was diluted 1:10 in Luria-Bertani broth with ampicillin, grown for 2 h, and then induced with 0.4 mM isopropyl-β-thiogalactopyranoside (IPTG) for 2 h at 37°C. Bacteria were pelleted and resuspended in 1/10 volume of phosphate-buffered saline (PBS), sonicated, and centrifuged in Eppendorf tubes at 13,000 rpm. These supernatants were either used directly for immunoprecipitation or incubated with glutathione (GSH)-agarose beads (Sigma). Swollen GSH-agarose beads were reacted for 20 min at room temperature with extracts containing the GST proteins at a ratio of 50 μl of beads per ml of extract. The coated beads were then washed three times in Z/p65 buffer (20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES; pH 7.7], 25 mM NaCl, 2.5 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol [DTT], 0.05% Nonidet P-40) and then used in GST precipitation experiments.

The Flag Z and Flag ZΔCOOH plasmids were introduced into *E. coli* DH5αF' (Gibco BRL), grown to log phase, and induced for 4 h with IPTG. Proteins were harvested first by incubation in 50 mM Tris-HCl (pH 8)–1 mM EDTA–100 mM NaCl–0.1 mM phenylmethylsulfonyl fluoride–1 mM sodium metabisulfite–1 mg of lysozyme per ml. Sodium deoxycholate was added to 16 mg/ml, and the lysates were incubated at 37°C for 10 min and then sonicated. The cellular debris was pelleted at $12,000 \times g$, and the supernatant was dialyzed against 25 mM HEPES–12.5 mM MgCl₂–100 mM KCl–30 μM phenylmethylsulfonyl fluoride–12.5 μM sodium metabisulfite–1 mM DTT–20% glycerol and then used directly for ^{32}P labeling. The Flag protein labeling reaction mixture contained 20 mM Tris-HCl (pH 7.5), 1 mM DTT, 10 mM NaCl, 12 mM MgCl₂, 2 μl of [γ - ^{32}P]ATP (Amersham Life Sciences; catalog no. PB.10168), 10 U of heart muscle kinase (Sigma; catalog no. P-2645), and 2 μl of crude bacterial extract containing Flag Z protein in a total volume of 30 μl (8). Proteins were labeled at 37°C for 1 h. In vitro transcription and translation for ^{35}S labeling reactions were performed as suggested by the manufacturer (Promega).

GST precipitations. Ten microliters of GST, GST-Z, or GST-p65-coated GSH-agarose beads was incubated with either 10,000 cpm of ^{32}P -labeled Flag protein or 10 μl of reticulocyte lysate containing ^{35}S -labeled Z or p65 protein in a 1-ml total volume of Z/p65 buffer. Following a 1-h room temperature incubation, the beads were washed five times with Z/p65 buffer and boiled in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) loading buffer. The proteins were then separated on a 10% denaturing polyacrylamide gel and submitted to autoradiography (enhanced with 1 M sodium salicylate for ^{35}S -labeled proteins).

To demonstrate a genuine DNA-independent interaction between Z and p65, the crude bacterial extracts containing GST fusion proteins and the reticulocyte lysates containing ^{35}S -labeled proteins were treated with DEAE-Sephacel prior to GST precipitation as previously described (1). As a marker for the removal of nonspecific DNA, protein extracts were spiked with a ^{32}P -labeled tracer probe containing the class I major histocompatibility complex NF-κB DNA-binding site (5)

FIG. 1. Z-induced transactivation of several EBV promoters is inhibited by NF- κ B p65. (A) Plasmid pBHRF1-CAT (3 μ g) was transfected into the HeLa epithelial cell line with various combinations of expression plasmids (each at 3 μ g) as follows (left to right): pBHRF1-CAT with the vectors pCMV4T and pHD1013; pBHRF1-CAT with pCMV4T and the Z expression vector CMV-Z; pBHRF1-CAT with the p65 expression vector pCMV-p65; pBHRF1-CAT with the p65 Δ expression vector pCMV4T-p65 Δ ; pBHRF1-CAT with pCMV-p65 and CMV-Z; and pBHRF1-CAT with pCMV4T-p65 Δ and CMV-Z. The p65 vector decreased Z transactivation of the BHRF1 promoter only to a moderate degree in comparison with the inhibition of Z by p65. (B) The BMRF1-CAT (pEA-BS-CAT) plasmid (5 μ g) was transfected into the HeLa epithelial cell line with various combinations of expression plasmids (each at 3 μ g) as follows (left to right): BMRF1-CAT with the vectors pCMV4T and pHD1013; BMRF1-CAT with pCMV-p65; BMRF1-CAT with the I κ B expression vector pCMV4T-I κ B; BMRF1-CAT with CMV-Z; BMRF1-CAT with pCMV-p65 and CMV-Z; BMRF1-CAT with pCMV-p65 and pCMV4T-I κ B; and BMRF1-CAT with pCMV-p65, CMV-Z, and pCMV4T-I κ B. As with the BHRF1 promoter, the p65 vector decreased Z transactivation of the BMRF1 promoter (fifth column). The addition of the I κ B expression vector overcame the inhibitory effect of p65 upon Z-induced transactivation of the BMRF1 promoter (seventh column). (C) The BHRF1-CAT plasmid (3 μ g) was transfected into the Raji B-cell line with various combinations of expression plasmids (each at 3 μ g) as follows (left to right): the vectors pCMV4T and pHD1013; pCMV4T-I κ B; pCMV4T and CMV-Z; and pCMV4T-I κ B and CMV-Z. Coexpression of I κ B with Z increased Z-induced transactivation of BHRF1-CAT fourfold above the level of transactivation due to Z alone in Raji cells. Error bars indicate the standard error of the mean.

and then equally divided for GST precipitation studies with extracts untreated or treated with DEAE-Sephacel.

Immunoprecipitations and immunoblotting. Approximately 10^7 HeLa cells per condition were transfected by electroporation with various combinations of Z and p65 expression vectors or the appropriate negative control vectors. At 24 h posttransfection, the cells were scraped from the dishes, washed twice with ice-cold PBS, resuspended in 250 μ l of Z/p65 buffer, and sonicated. The cellular debris was removed by centrifugation, and 100 μ l of supernatant was reacted with either 15 μ l of normal rabbit serum or 15 μ l of anti-p65 serum (Santa Cruz Biotechnology; catalog no. SC-109) in a total volume of 500 μ l of Z/p65 buffer. The reactions were rocked for 1 h at 4°C before 20 μ l of preswollen protein A-Sepharose beads (Pharmacia) was added. Samples were again rocked for 1 h at 4°C, washed three times with Z/p65 buffer, and boiled in SDS-PAGE loading buffer. The proteins were then separated on a 10% denaturing polyacrylamide gel and transferred to nitrocellulose (Schleicher & Schuell). Ten microliters of each original cellular extract (directly loaded to demonstrate protein levels prior to immunoprecipitation) were also separated on the denaturing gel and transferred.

Approximately 5×10^5 P3HR1 cells were equally divided and either induced with 5 mM sodium butyrate plus 30 ng of 12-O-tetradecanoylphorbol-13-acetate (TPA) per ml or left uninduced. After 34 h, the cells were pelleted, washed twice with ice-cold PBS, resuspended in 500 μ l of Z/p65 buffer, and sonicated. The cellular debris was removed by centrifugation, and supernatant containing 1.9 mg of cellular protein was reacted with either normal rabbit serum (3 μ g of protein) or anti-p65 serum (3 μ g) (Santa Cruz Biotechnology) in a total volume of 500 μ l of Z/p65 buffer. Hereafter, the immunoprecipitation was performed exactly as for the HeLa cell experiment.

Immunoblotting was performed in PBS with 3% powdered milk with a 1:10 dilution of monoclonal antibody BZ.1 (a gift of Alan Rickinson) (74) and a 1:2,000 dilution of goat anti-mouse kappa-chain horseradish peroxidase-conjugated serum (Southern Biotechnology Associates, Inc.; catalog no. 1050-05), followed by enhanced chemiluminescence performed as recommended by the manufacturer (Amersham Life Sciences). Rabbit anti-p65 serum was used at 1:2,000 in 3% milk-PBS, and the secondary antibody was donkey anti-rabbit horseradish peroxidase-conjugated serum (Amersham Life Sciences) at 1:3,000, in the procedure described above for anti-Z immunoblotting.

RESULTS

The p65 component of NF- κ B inhibits the ability of Z to activate EBV early promoters in HeLa cells. In search of a mechanism for the cell type specificity of Z transactivation function, we first studied the ability of p65 to interfere with Z-induced transactivation of the BHRF1 early promoter of EBV (42). In HeLa cells, the I κ B proteins physically bind to the p65 component of NF- κ B, thereby rendering NF- κ B inactive (3). We introduced an expression vector for p65 into HeLa cells to test the ability of transiently overexpressed p65 to inhibit transactivation of the BHRF1 promoter by Z. In the HeLa epithelial cell line, Z by itself efficiently activated the BHRF1 promoter (Fig. 1A). Although p65 alone had little effect on BHRF1 activity, coexpression of p65 with Z totally inhibited transactivation of BHRF1 by Z. Western blotting (immunoblotting) confirmed that this functional inhibition of Z is not caused by decreased expression of Z through inhibition of the CMV promoter driving Z expression in the presence of p65 (see Fig. 8A).

A transforming, alternatively spliced form of p65, called

p65 Δ , lacks amino acids 222 to 231 of p65 (58). The deletion within the Rel domain of this variant p65 protein is known to interfere with multimerization such that the p65 Δ protein is unable to homodimerize or heterodimerize with NF- κ B p50 and only weakly interacts with p65 (58). We examined the effect of this multimerization-defective form of p65 on Z. As shown in Fig. 1A, p65 Δ had a less profound effect upon the Z-induced transactivation of BHRF1-CAT, suggesting that amino acid residues 222 to 231 (within the Rel homology domain) of p65 are necessary for efficient inhibition of Z by p65.

We next tested whether transactivation of a second EBV early promoter, BMRF1, by Z might also be inhibited by p65. Our previous studies have demonstrated that the BMRF1 promoter, which controls the expression of the early antigen-diffuse polymerase cofactor (40), responds to Z in a cell-type-specific manner (29, 32). In the lymphoid cell lines Jurkat and Louckes, Z has very little effect on the BMRF1 promoter; however, in the epithelial cell line HeLa, Z can efficiently activate BMRF1. Expression of the Z protein alone produced the expected transactivation of the BMRF1 promoter linked to CAT (Fig. 1B). When a p65 expression vector was introduced along with the Z expression vector, activation of BMRF1 by Z was inhibited by 88%. Overexpression of I κ B α with Z and p65 resulted in reversal of the p65-induced inhibition of Z. Additionally, gel retardation studies indicate that while Z alone can bind to the BMRF1 promoter (32), p65 alone does not (29a), making it unlikely that p65 is merely competing with Z for DNA binding sites within this promoter.

Endogenous NF- κ B p65 expression inhibits Z function in the B-cell line Raji. In B cells, I κ B does not efficiently sequester NF- κ B into the cytoplasm, leaving a portion of NF- κ B free to remain nuclear in localization and functional (39, 63). We tested whether overexpression of I κ B in the B-cell line Raji might inactivate p65, allowing Z to function more efficiently. Like other mature B cells, Raji cells are known to contain constitutive NF- κ B-binding activity in the nucleus (23). Although expression of Z alone resulted in some transactivation of BHRF1 in Raji cells (Fig. 1C), overexpression of I κ B α increased the ability of Z to transactivate the BHRF1 promoter fourfold (Fig. 1C, last column). The positive effect of I κ B in Raji cells is presumably due to overexpression of I κ B α and the sequestration of endogenous p65 into the cytoplasm, thereby eliminating p65-induced inhibition of Z function in the nucleus. However, a more indirect effect of I κ B α upon Z cannot be ruled out.

Z and p65 physically associate with one another. One possible means by which p65 inhibits Z is through a direct contact between the proteins. To test whether such an interaction takes place, we performed precipitation experiments using a GST-p65 fusion protein. Full-length Z protein labeled to very high specific activity with 32 P (Fig. 2A) was not precipitated by GSH-agarose beads alone or by beads coated with GST protein (Fig. 2B). However, labeled Z protein was efficiently precipitated by using GST-p65 linked to GSH-agarose beads (Fig. 2B).

To begin to determine which portion of Z is necessary for the contact with the p65 protein, a truncated form of Z (Flag Z Δ COOH) in which the carboxy-terminal 74 amino acids of Z had been deleted was 32 P labeled and compared with full-length Z for the ability to bind to GST-p65 (Fig. 2C). In contrast to the full-length Z protein, the carboxy-terminal truncated form of Z was unable to associate with p65. These last 74 amino acids of Z contain the basic DNA-binding domain and the coiled-coil dimerization domain.

Because Z and p65 are both DNA-binding proteins, the

possibility exists that they each nonspecifically interact with contaminating DNA present in bacterial or reticulocyte lysates. This genomic DNA could then serve as a bridge between two DNA-binding proteins, creating the appearance of a protein-protein interaction (37). Therefore, we treated the lysates containing Z and p65 with DEAE-Sepharose to remove any DNA present (1). Figure 3B demonstrates that DEAE-Sepharose treatment totally eliminates a tracer probe (to which p65 is known to bind) from all lysates used in Fig. 3A. In these DNA-free lysates, 35 S-labeled Z and p65 retain the ability to interact with GST-p65 and GST-Z, respectively (Fig. 3A).

The dimerization domain of Z is required for the Z-p65 interaction. To ascertain further which domain of Z is needed for binding directly to p65, each of a series of truncated Z proteins was linked to GST (Fig. 2A), and each fusion protein was tested for the ability to precipitate 35 S-labeled p65. The Z proteins lacking a transactivation domain retained the ability to precipitate p65 (Fig. 2D, GST Z Δ 1-79 and GST Z Δ 1-133). However, specific deletion of the dimerization domain was shown to eliminate the Z-p65 interaction. These data suggest that the coiled-coil domain of Z is required for the interaction with p65.

To further establish that the dimerization domain of Z is required for the interaction with p65 and to determine which amino acid residues within the coiled-coil are critical, various mutant forms of the Z protein were tested for the ability to multimerize with p65 (Fig. 4). These mutant Z proteins all contain two site-directed mutations within the dimerization domain (mutants are numbered for the amino acids which were mutated, and letters indicate which residues replaced the wild-type amino acids). The abilities of the GST-Z and GST-p65 fusions to complex with the various Z mutants were compared. Mutants Z205R/206D and Z209R/216E as well as wild-type Z protein all demonstrated the capacity to homodimerize with GST-Z. These same Z proteins interacted efficiently with GST-p65. The remaining Z mutants were found to be incapable of efficient homodimerization (as was previously reported [20]) and also failed to interact with GST-p65.

Mapping the portion of p65 required for the interaction with Z. To similarly determine which part of the p65 protein is needed for contact with Z, we tested the ability of a series of 35 S-labeled mutant and truncated p65 proteins to interact with the GST-Z fusion protein (Fig. 5A). All of these in vitro-translated p65 proteins were present in similar amounts (data not shown). None of the p65 proteins reacted nonspecifically to the GST protein linked to GSH-agarose beads (Fig. 5B). Wild-type p65 and a mutant p65 form, p65 Δ NLS, both were efficiently precipitated by GST-Z. The p65 Δ NLS protein, which contains a four-amino-acid substitution within a nuclear localization signal and is incapable of interacting with the I κ B α protein (6), still interacted efficiently with Z. Deletion of the last 238 amino acids of p65, which contain the transactivation domain, did not affect the Z-p65 interaction. However, deletion of the region between amino acids 286 through 313 destroyed the ability of p65 to interact with Z. Although the portion of p65 between amino acids 285 and 314 is needed for the binding of p65 to both I κ B α and Z, the precise amino acids which are required are not identical, since the p65 Δ NLS mutant can interact with Z but not I κ B α .

We also tested whether the p65 Δ protein (which lacks 10 amino acids within the Rel homology domain of p65 and was incapable of fully inhibiting transactivation of the BHRF1 promoter by Z) might be incapable of efficient interaction with GST-Z. As shown in Fig. 6 (lanes 5, 8, and 11), GST-Z efficiently precipitated 35 S-labeled, in vitro-translated Z and p65 proteins. However, although equal amounts of p65 and

FIG. 2. The direct physical interaction between Z and p65 requires the carboxy terminus of Z. (A) Map of the Flag Z protein containing all 245 amino acids of Z linked to an N-terminal Flag antibody epitope (F) with a kinase site for 32 P labeling (8). The transactivation (TA), DNA-binding (DNA), and coiled-coil dimerization (DIM) domains of Z are also shown. The Flag Z Δ COOH protein lacks the last 74 amino acids of Flag Z. A map of GST Z which contains the GST protein from pGEX-3X (Pharmacia) linked to the wild-type full-length Z protein is shown along with various truncated Z proteins which have also been linked to GST. The truncated Z proteins are named by the amino acid residues which have been removed. (B) The GST protein or the GST-p65 fusion protein was linked to GSH-agarose beads, and these beads were incubated with Flag Z protein which had been labeled with 32 P to a high specific activity. Precipitated bead-protein complexes were washed five times and loaded on an SDS-polyacrylamide gel. (C) To map the portion of Z necessary for the Z-p65 interaction, a truncated form of Z was also tested in GST precipitation experiments performed as described for panel B. The first two lanes show 5,000 cpm of 32 P-labeled Flag Z and Flag Z Δ COOH loaded directly on the SDS-polyacrylamide gel. Flag Z was reacted with GSH-agarose beads coated with GST protein or with GST-p65 protein, as indicated. On the same gel, Flag Z Δ COOH was reacted with GSH-agarose beads coated with GST protein, or with GST-p65 protein, as indicated. (D) To determine which domains of the Z protein are necessary for the Z-p65 physical interaction, GST fusion proteins containing various truncated forms of the Z protein were linked to GSH-agarose beads and incubated with in vitro-translated, 35 S-labeled p65 protein. The carboxy-terminal 112 amino acids (GST Z Δ 1-133) were sufficient for the interaction with p65, yet the truncated Z protein lacking the coiled-coil dimerization domain failed to precipitate p65. The GST-Z fusion proteins were present on the beads in similar amounts (data not shown). The p65 protein retained by GST-Z appeared lighter than the previous experiments because of the use of 35 S-labeled protein (versus 32 P-labeled Z protein in panels B and C).

p65 Δ protein were used (Fig. 6, lanes 2 and 3), significantly more p65 than p65 Δ protein bound to Z (Fig. 6, lanes 8 and 11). This result provides further evidence that the Rel homology domain of p65 is required for the interaction with Z.

Z and p65 can be coimmunoprecipitated in vitro and in vivo. We used coimmunoprecipitation as a second method to confirm the physical interaction between Z and p65. In the first set of experiments, the source of p65 protein was the GST-p65 fusion protein and the source of Z was 32 P-labeled Flag Z (Fig. 2A). Rabbit anti-p65 serum, as expected, could specifically precipitate 35 S-labeled p65, whereas the preimmune rabbit serum did not (Fig. 7, last two lanes). Preimmune serum did

not precipitate Z in the presence of GST-p65 or the negative control GST protein alone. Anti-p65 serum could not precipitate Z in the absence of p65, but when GST-p65 was added with 32 P-labeled Z, the Z was coprecipitated.

We also performed coimmunoprecipitations to confirm that an interaction between Z and p65 occurs in vivo, using HeLa cells transfected exactly as those used for Fig. 1. Rabbit anti-p65 serum added to extracts from HeLa cells which had been transfected with expression vectors for Z and for p65 coprecipitated Z protein (Fig. 8A, lower panel, lane 4). Even in the absence of transfected p65, the Z protein produced from the transfected Z expression vector was precipitated through

FIG. 3. Z and p65 interact in a DNA-independent manner. (A) 35 S-labeled Z and p65 proteins were reacted with the GST protein (pGEX-3X) or the GST-Z and GST-p65 fusion proteins both before (Pre-Cleared) and after treatment of all protein extracts with DEAE-Sephacel. Load lanes contain 35 S-labeled Z and p65 proteins directly loaded onto the protein gel as markers. (B) All of the extracts used in panel A were treated with DEAE-Sephacel to remove DNA present in the bacterial extracts and reticulocyte lysates. A marker 32 P-labeled probe is shown for all extracts before (Pre-Cleared) and after (DEAE-Sephacel) treatment, demonstrating complete removal of any DNA present in the extracts.

its interaction with endogenous p65 protein (Fig. 8A, lower panel, lane 2). Z protein was not nonspecifically precipitated by normal rabbit serum. These data imply that the direct physical interaction between Z and p65 may mediate the functional inhibition of Z transactivation by p65.

Even stronger evidence that an interaction between Z and p65 occurs *in vivo* was demonstrated by using the endogenous, virally produced Z protein from the EBV-positive cell line P3HR1. These cells were treated with sodium butyrate and TPA to induce the expression of Z protein. Anti-p65 serum coimmunoprecipitated endogenous p65 and Z protein from induced cells but not from latently infected, uninduced P3HR1 cells which do not express Z protein (Fig. 8B). Thus, the Z and p65 proteins appear to associate through a direct physical interaction both *in vitro* and *in vivo*.

DISCUSSION

In this report, we have demonstrated that the EBV Z protein, which triggers the switch from latent to lytic infection, can interact directly with the p65 component of NF- κ B. This interaction is mediated through the coiled-coil dimerization domain of Z and the Rel homology domain of p65. We demonstrate that p65 inhibits the ability of Z to transactivate early EBV promoters. Therefore, B-cell-specific nuclear expression of NF- κ B or other Rel proteins may be one mechanism of promoting EBV latency in B cells.

Heteromultimerization not only within families of transactivators but also between whole classes of transactivators

FIG. 4. The same amino acids of Z which are important for homodimer formation are required for the Z-p65 interaction. (A) GST precipitation experiments were performed similarly to those in Fig. 2. Either reticulocyte lysates containing no Z RNA (– Retic. Lysate) or *in vitro*-translated, 35 S-labeled Z wild-type (Z WT) or Z mutant proteins were reacted with GSH-agarose beads coated with GST protein or with GST-Z protein, as indicated. Mutant forms of the Z protein are named for the locations of the amino acid residues of Z that have been mutated and indicate the amino acids which now are present in place of the wild-type amino acids. (B) The 35 S-labeled Z proteins were incubated with GSH-agarose beads coated with GST protein or with GST-p65 protein, as indicated. (C) The various forms of 35 S-labeled Z proteins were loaded directly on an SDS-polyacrylamide gel to demonstrate that similar counts per minute of protein were used in all of the conditions in panels A and B.

increases the complexity in regulation of gene expression. Recently, members of the bZIP family have been shown to directly interact with a number of diverse proteins, including helix-loop-helix-containing transcription factors (7, 8), steroid receptors (61), and the TATA-binding protein (41). Of particular relevance to the Z-p65 interaction described here are the recent reports showing that another bZIP protein (NF-IL6) can interact directly with both NF- κ B p50 (38) and p65 (67). Interestingly, as is the case with the Z-p65 interaction, the p65–NF-IL6 interaction requires the Rel domain of p65 and the leucine zipper of NF-IL6 (67).

The leucine zipper domain of Z and the Rel domain of p65 are responsible for homodimerization of the respective proteins (10, 20, 35, 52, 58) but also appear to be important mediators of interactions with other proteins. We found that the Z mutants which disrupt homodimerization by interfering with the hydrophobic face of the Z coiled-coil domain (20) are also unable to interact with p65 (Fig. 4). It is possible that the hydrophobic face of the zipper which normally contacts a second Z monomer contacts p65 instead, thereby excluding Z from forming a functional binding protein or perhaps changing the specificity of DNA binding by Z. Alternatively, it is possible that while the Z homodimerization domain is necessary for the Z-p65 interaction, the interaction between Z and p65 is

FIG. 5. Mapping the region of the p65 protein necessary for the interaction with Z. (A) Maps of wild-type (WT), mutant, and deletional forms of the p65 protein (translated in vitro and 35 S labeled). To generate the p65 Δ NLS protein, a four-amino-acid mutation from KRKR to DQNQ was introduced in the nuclear localization signal of p65. Carboxy-terminal deletion proteins were named by using the number corresponding to the terminal amino acid residue. (B) The panel of p65 proteins was reacted with GSH-agarose beads coated with GST protein or with GST-Z (wild-type) protein, as indicated. Each p65 protein was expressed in vitro at the same level, and the arrows show where loaded aliquots of the indicated proteins ran on the gel.

mediated through another, yet unmapped portion of the Z protein.

As is the case for the p65 interaction with NF-IL6, the Rel portion of p65 was also found to be essential for interaction with Z. The p65 protein has been noted to contain a putative leucine zipper, the disruption of which interferes with the function of the p65 activation domain (58). Although this motif theoretically might dimerize with Z, the leucine zipper is located in the carboxy-terminal portion of p65 which is dispensable for binding to Z. Additionally, the p65 Δ protein (which lacks amino acids 222 to 231 of p65) contains an intact leucine zipper, yet p65 Δ interacts less efficiently with Z than does full-length p65 protein.

Interactions of viral proteins with NF- κ B are not without precedent; however, the physical binding of Z to p65 is a novel mechanism of viral interaction with NF- κ B. Functional NF- κ B binding sites are found in the regulatory regions controlling the expression of important proteins encoded by several viruses (12, 46, 47, 55, 60). The Tax protein of human T-cell leukemia virus type I can interact directly with the p105 precursor of

FIG. 6. The same portion of p65 which is important for multimerization is required for efficient protein-protein interaction with Z. In vitro-translated, 35 S-labeled wild-type Z, wild-type p65, and p65 Δ (the last protein in Fig. 5A) were directly loaded in lanes 1 to 3 at 1/25 the amount used in GST precipitation conditions shown in lanes 4 to 12. GSH-agarose beads coated with the GST, GST-Z, or GST-p65 proteins were reacted with 35 S-labeled Z (lanes 4 to 6), p65 (lanes 7 to 9), or p65 Δ (lanes 10 to 12). The p65 Δ protein, which multimerizes poorly with native p65 (illustrated by the fold decrease from lane 9 to lane 12) interacts even less with Z (note the fold decrease from lane 8 to lane 11).

NF- κ B p50 (28). The human immunodeficiency virus type 1 protease processes the p105 precursor to a functional p50 product (54). Numerous viruses exhibit the capacity to transactivate promoters through NF- κ B sites (reviewed in reference 2). Interestingly, the EBV LMP1 protein, which is expressed in latently infected B cells, also activates both the human immunodeficiency virus long terminal repeat (25) and the cellular A20 zinc finger protein (36) through NF- κ B sites. The induction of NF- κ B activity by LMP1 could potentially strengthen the latent state by inhibiting Z protein function.

In contrast to the positive effect that NF- κ B has on viral replication in the preceding examples, EBV appears to use

FIG. 7. The Z protein can be coimmunoprecipitated with p65. In vitro, bacterially expressed GST-p65 protein (or GST protein alone as a negative control) was reacted with 32 P-labeled Flag Z protein (Fig. 2A) and then incubated with rabbit preimmune serum or rabbit anti-p65 serum, as shown in the conditions specified above lanes 1 to 4. The 32 P-labeled Z protein was added in equal amounts to all of the reactions shown in lanes 1 to 4; however, Z was precipitated only in the presence of p65 protein and anti-p65 antibody (lane 4). Equal counts of in vitro-translated, 35 S-labeled p65 protein were added to lanes 5 and 6 to demonstrate that the anti-p65, but not the preimmune serum, can immunoprecipitate p65 protein.

FIG. 8. The Z and p65 proteins form a complex in vivo. (A) HeLa cells were transfected with 3 μ g each of pHD1013 plus pCMV-4T (lane 1), CMV-Z plus pCMV-4T (lane 2), pCMV-p65 plus pHD1013 (lane 3), or pCMV-p65 plus CMV-Z (lane 4). Whole cell lysates of the transfected HeLa cells were divided and either loaded directly onto an SDS-polyacrylamide gel and then subjected to Western transfer (upper two panels) or first immunoprecipitated with normal rabbit serum (third panel) or rabbit anti-p65 serum (fourth panel) and then subjected to immunoblotting. Anti-p65 rabbit serum was used to probe the first immunoblot, and an anti-Z monoclonal antibody was used to probe the last three blots. The anti-p65 serum, but not the normal rabbit serum, coimmunoprecipitated p65 and Z (compare lane 4 for the bottom two panels). The low level of endogenous, untransfected p65 present in the HeLa cells can also complex with Z (see the faint Z band which has been coimmunoprecipitated in lane 2, lower panel). (B) Whole cell lysates of P3HR1 cells (both uninduced or induced with sodium butyrate and TPA) were divided and either loaded directly onto an SDS-polyacrylamide gel and then subjected to Western transfer (two right panels) or first immunoprecipitated (IP) with normal rabbit serum or rabbit anti-p65 serum (lower left panel) and then subjected to immunoblotting. Anti-p65 rabbit serum was used to probe the right upper immunoblot, and an anti-Z monoclonal antibody was used to probe the lower two blots. The level of p65 protein was unchanged with induction (upper right panel). As expected, the level of Z protein increased dramatically following induction (lower right panel). Anti-p65 serum (not normal rabbit serum) coimmunoprecipitated Z from the induced, but not from the uninduced, P3HR1 cell extracts.

NF- κ B as an inhibitor of productive infection. Infection of humans by EBV primarily involves epithelial cells and B cells (33). In epithelial cells, the virus replicates efficiently, producing virions which are shed into the saliva (66). In B cells, EBV exists primarily in a latent state in which little if any Z gene

expression occurs (44). However, even a small amount of "leaky" transcription in B cells might be expected to irreversibly launch the lytic viral cascade, since Z activates its own expression (19, 71) as well as the expression of early EBV promoters (9, 11, 13, 14, 16, 30, 31, 42, 43, 64, 71). The presence of NF- κ B in the nuclei of B cells could serve to prevent such leaky expression of Z from initiating full viral replication. On the other hand, elevated expression of I κ B could potentially lead to disruption of latency in B cells.

Interestingly, the phorbol ester TPA can serve both as an inducer of NF- κ B activity within the nucleus of some cells (62) and as an inducer of Z expression (and EBV replication) in EBV-infected B cells (77). While these observations would appear to contradict our model of NF- κ B inhibition of Z function, the effect of TPA on NF- κ B activity is transient, reaching a maximum in several hours and then returning to baseline by 8 to 24 h (49, 68). On the other hand, induction of EBV productive infection by TPA is very slow, with an onset following 2 to 3 days of exposure and reaching a maximum in 5 to 8 days (77). Hence, EBV is induced by TPA after NF- κ B activity has fallen to preinduced levels. It is therefore likely that the disruption of EBV latency by TPA is mediated through a different mechanistic pathway than that which mediates TPA induction of NF- κ B activity.

The cellular regulation of EBV latency is likely to be very complex and involve multiple mechanisms. In addition to p65, several other cellular proteins have been recently shown to negatively regulate Z transactivation function through direct protein-protein interactions. The cellular tumor suppressor protein p53 inhibits Z-induced transactivation of both the BMRF1 and BHFR1 promoters (76). This inhibition, which involves a direct physical interaction between Z and p53, requires the coiled-coil dimerization domain of Z and the carboxy-terminal portion of p53 (76). In a similar manner, the retinoic acid receptors, RAR α and RXR α , repress the ability of Z to transactivate the BMRF1 promoter and can physically associate with the Z protein (65). In addition to negative control of Z transactivation function, control of BZLF1 transcription in different cell types must play a role in determining whether EBV remains latent versus productive. In this regard, we have recently shown that the BRLF1 promoter (one of two EBV promoters driving Z expression) is constitutively active in epithelial cells but inactive in B cells (75).

Although a number of cellular factors have been shown to inhibit Z transactivation function, in certain circumstances disruption of viral latency can occur, and therefore mechanisms whereby these negative regulators of Z function can be circumvented must also exist. Clearly, if Z transcription is induced to a sufficient level, then Z can further activate its own transcription and thereby produce enough protein to overcome the effect of negative cellular regulators. Z transactivator function in certain lymphocytic cell lines is increased by the EBV BRLF1 immediate-early gene product (29) or the *c-myc* oncogene product (32). In addition, the interactions between Z and cellular proteins may well be influenced by Z phosphorylation, since there is recent evidence that Z is phosphorylated in vivo and that dephosphorylation of Z is associated with the disruption of viral latency (17). In any event, it is now clear that the regulation of EBV latency is cell type specific and involves interactions with numerous viral and cellular proteins.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants P01-CA19014, K04-CA01711, and F32-AI08408 from the National Institutes of Health and grant MV-532 from the American Cancer Society.

We thank Amer Beg, Alain Sergeant, Craig Rosen, Paul Farrell, Erik Flemington, and Samuel Speck for gifts of plasmids and Alan Rickinson for monoclonal antibody BZ.1.

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