Nucleic Acids Research, 1995, Vol. 23, No. 10 1729–1736

Physical and functional interaction of the Epstein–Barr virus BZLF1 transactivator with the retinoic acid receptors RARα and RXRα

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Received February 6, 1995; Accepted February 23, 1995

ABSTRACT

Epstein–Barr virus (EBV) reactivation, indicated by induction of EBV early antigens from latently infected lymphoid cell lines by phorbol esters, is inhibited by retinoic acid (RA). Viral reactivation, which is triggered by the immediate-early BZLF-1 (Z) viral gene product, is repressed by retinoic acid receptors (RARs) RARα and RXRα. These proteins negatively regulate Z-mediated transactivation of the promoter for an EBV early gene product, early antigen-diffuse (EaD). Here we confirm a direct physical interaction between the AP1-like protein Z and RXRα and map the domains of interaction in the Z protein and RXRα. The domain required for homodimerization of Z is separate from that required for its interaction with RXRα. Z also has the effect of repressing activation of an RAR-responsive cellular promoter (BRE). Point mutants in the dimerization domain of Z unable to interact with RXRα do not repress RXRα-mediated transactivation of BRE, the promoter for RARβ, which suggests that interaction between the two proteins is required for this repressor effect. The domain of RXRα required for interaction with Z has been mapped, and is again separate from that required for homodimerization. These results indicate that a 'cross-coupling' or direct interaction between Z and RARα and RXRα can modulate the reactivation of latent EBV infection and suggest that, reciprocally, the viral protein Z may influence cellular regulatory pathways.

INTRODUCTION

Epstein–Barr virus (EBV) is a human herpesvirus that infects lymphocytes and certain epithelial cells and is associated with several malignancies. EBV is prominently associated with B-cell lymphomas and rare T-cell malignancies as well as nasopharyngeal and parotid carcinomas. In oral hairy leukoplakia in immunosuppressed persons, EBV replicates rapidly in the differentiated epithelial cells of the tongue. Infection of B-lymphocytes is predominantly latent and results in lymphoproliferation. Infection of epithelial cells can be latent, as in nasopharyngeal carcinoma, but usually leads to complete viral replication resulting in cytolysis. In cell culture, several factors can induce the latent virus into its productive phase, such as TPA, sodium butyrate, surface anti-IgG cross-linking and nucleoside analogs, all of which mediate their effect through the EBV immediate-early BZLF-1 protein Z (also called ZEBRA, Zta, EB-1). Z serves as a trigger to disrupt latency by initiating a cascade of events that includes transactivation of other regulatory proteins, BRLF-1 (R) and BMLF-1 (M), which together with Z activate early genes; DNA replication and late gene expression follow (1–3). Therefore, Z is a central target for interaction with cellular proteins, some of which may abrogate Z-mediated transactivation and potentially curb cytolysis.

Z, a member of the basic leucine-zipper family, transactivates the viral early promoters by binding to upstream binding sites, Z-response elements (ZREs) (1–9) that are similar and sometimes identical to AP1 sites (1,2,5,6,8–11). Interaction of cellular or viral factors results in modulation of Z action to produce synergistic transactivation or repression of early promoters. For instance, the viral immediate-early protein R can synergize with Z to transactivate early promoters (12) as does the cellular factor, c-*myb* (13). Recently p53 and p65 have been shown to interact with Z (14,15) and repress Z-mediated transactivation. Thus there are several cellular regulators, some of which tend to counter reactivation of this normally latent infection. In addition, a recently described viral gene product, RAZ, which acts as a transdominant repressor, can abort Z-mediated reactivation in latently infected cells (16).

We have previously shown that the retinoic acid receptor, RARα, and the retinoid X receptor, RXRα, repress Z-mediated transactivation of the EBV early promoter, BMRF1 (17). RARs and RXRs are members of the steroid hormone receptor superfamily that differ in their ligand-binding domains (18). Retinoic acid (RA) is the ligand for RAR whereas a metabolic derivative of RA, 9-*cis* RA, is the ligand for the RXR receptor (19). The receptors form homo and heterodimers which transcriptionally activate target genes by binding to *cis*-acting elements

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called retinoic-acid response elements (RARE). While RARs and RXRs are effective activators of some genes, they are also known to repress several genes by different mechanisms (20,21). Retinoic acid receptors not only repress activation of viral genes by Z, but conversely, Z can also repress RARα and RXRα-mediated transactivation of a cellular promoter (BRE) for the RARβ gene. RXRα prevents Z from binding to an AP1 site in the BMRF1 promoter and, reciprocally, Z inhibits RARα from binding to a RARE in the RARβ promoter (17). Thus these cellular factors can modulate viral activators, and conversely, the viral AP1-like protein can itself affect cellular circuitry. Although steroid hormone receptors, particularly RAR and glucocorticoid receptor (GR), have an antagonistic relation with the AP1 family of proteins (22), both inhibiting AP1 from binding to its cognate DNA sequences (22), a direct physical interaction between the zinc-finger-containing retinoic acid receptors and the leucine-zipper-containing AP1 protein(s) has not been shown.

Here we demonstrate such an interaction between both RXRα and RARα and the AP1-like Z protein *in vitro* with glutathione-*S*-transferase fusion proteins. We have mapped the domain in RXRα that interacts with Z and found that it is different from that required for homodimerization of RXRα. Using point mutations in the dimerization domain of Z, we demonstrate that the region required for homodimerization of Z is separate from that required for interaction with RXRα. Corroborating the *in vitro* data are the functional effects observed *in vivo*, namely, that point mutants of Z which do not interact physically with RXRα also do not repress RXRα-mediated transactivation.

MATERIALS AND METHODS

Recombinant plasmids

The plasmid pGEX-Z was a generous gift from David Gutsch and Shannon Kenney (University of North Carolina at Chapel Hill). The clones pGEX-RXRα, pRshRARα and pRshRXRα were generous gifts from Ronald Evans. The plasmids with point mutations in the Z open reading frame (ORF) used to make *in vitro*-translated proteins were generous gifts from Eric Flemington. All the Z mutants were subcloned into pHD1013 for use in transfected cells. The RXRα ORF was subcloned as an *Eco*RI fragment into pBS (Stratagene). The GST-Z fusion protein contains the entire Z ORF cloned downstream of the glutathione transferase gene into the pGEX3X vector (Pharmacia). Constructs containing deletions in the Z ORF removing either 86 N-terminal amino acids (ZΔ 1–86), amino acids 25–29 (ZΔ 25–29), amino acids 140–227 (ZΔ 140–227) or amino acids 200–227 (ZΔ 200–227) as well as BMRF1-CAT (gifts from Shannon Kenney) were also used (13).

GST-fusion-protein affinity chromatography

The GST-Z and GST-RXRα were expressed as previously described (14,23). The ability of ^{35}S-labelled *in vitro*-translated RXRα to interact with GST-Z was analyzed by affinity chromatography. GST-Z fusion protein bound to beads was incubated with radiolabelled RXRα in reaction buffer (17) for 1 h at 37°C, washed, pelleted and boiled in Laemmli buffer. Bound proteins were resolved by SDS/PAGE on 10% gels and visualized by autoradiography.

DNA transfections

Plasmid DNA was amplified in *Escherichia coli* and purified through two sequential cesium chloride gradients or by Qiagen columns. DNA was transfected into EBV$^-$ lymphoblastoid cells (Loucke) by electroporation (24) using 10 μg DNA and 10^7 cells/condition. The cells were shocked at 1500 V with a Zapper electroporation unit (Medical Electronics Shop, University of Wisconsin). Twenty-four hours before transfections, cells were transferred to phenol-red-free RPMI 1640 medium supplemented with 5% (v/v) charcoal-stripped serum (CSS). Transfections were done in complete RPMI-1640 (10% v/v FCS), and then cells were returned to medium containing 5% CSS and incubated for 48 h at 37°C in 5% CO_2. Retinoic acid (RA) (1 μM) was added 24 h after transfection.

Promoter-reporter assays

Cell extracts were prepared 48 h after transfection and 60 μg of the cell extract was incubated with ^{14}C-chloramphenicol in the presence of acetyl CoA at 37°C as described previously (25). The percentage acetylation of chloramphenicol was quantitated by separating the acetylated products by thin-layer chromatography, visualized by autoradiography, and quantitated by scintillation counting of excised spots or by scanning on a Molecular Dynamics PhosphorImager.

Construction of RXRα mutants

The 3′ deletion mutants of RXRα were constructed by linearizing pBSRXRα with *Stu*I (1463) (deletes 36 amino acids) *Bss*HII (1185) (deletes 66 amino acids) or with *Bam*HI (777) (deletes 227 amino acids). Mutants spanning the region between *Bss*HII and *Bam*HI were obtained by PCR amplification with a 5′ primer containing the T7 promoter sequence 5′-CTCGAGAATTGTAATACGACTCACTATAGGG-3′ and 3′ primers at different positions as follows: 5′-CTCGAGCCCTGTCAAAGATGGCGCCC-3′ (1101), 5′-CTCGAGGGGAGAAGGAGGCGATGAGCA-3′ (999) and 5′-CTCGAGCTCCACCAGGGTGAAAAGC-3′ (899). The template was a gel-purified DNA fragment of pBSRXRα containing the intact RXRα ORF downstream of the T7 promoter. The PCR products were gel-purified and then used in *in vitro* transcription-translations to make a ^{35}S-methionine-labeled protein.

DNA-binding studies

A 17 bp oligonucleotide containing the AP1 sequence was end-labeled with the use of T4 polynucleotide kinase and [γ-^{32}P]ATP and used as probe for binding with *in vitro*-translated Z or Z mutants for 20 min at room temperature. Binding reactions were performed in buffer containing 250 mM Hepes, pH 7.9, 700 mM KCl, 5 mM EDTA, 10 mM DTT and 5% NP-40. Protein–DNA complexes were resolved by PAGE on 5% gels in 0.5 × TBE (1 × TBE + 0.09 M Tris-borate/0.002 M EDTA) and the dried gel was exposed with an intensifying screen at –70°C on Kodak XAR film.

RESULTS

Retinoic acid receptors RARα and RXRα bind to the dimerization domain of Z

We have shown that RARα and RXRα are repressors of Z-mediated transactivation of the EBV early promoter, BMRF-1. RXRα interferes with Z binding to the BMRF1 promoter and,

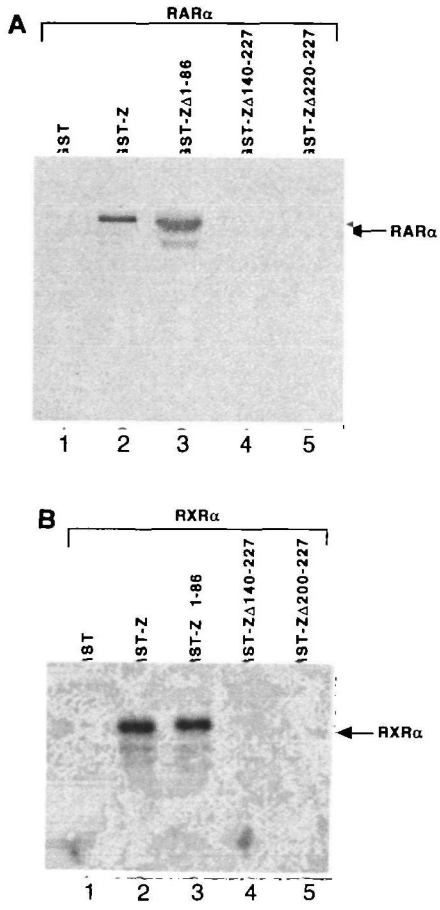

Figure 1. Dimerization domain of Z is required for the interaction with RXRα and RARα. (**A**) For dimerization experiments, ^{35}S-labeled RARα protein (10 000 c p m) was incubated with GST-fusion proteins bound to beads in reaction buffer (10 mM Tris, pH 8 0, 40 mM KCl, 0 05% NP-40, 6% glycerol, 1 mM DTT, 0 2 μg poly dI/dC) for 1 h at 37°C, washed, pelleted and boiled in Laemmli buffer Bound proteins were resolved on SDS/10% PAGE and visualized by autoradiography The arrow indicates the position of ^{35}S-labeled RARα protein retained by GST protein alone (lane 1) or GST-fusion proteins, GST-Z (lane 2), GST-ZΔ 1–86 (lane 3), GST-ZΔ 140–227 (lane 4) and GST-ZΔ 200–227 (lane 5), GST-Z (lane 2) or GST-RXRα (lane 3) (**B**) The same GST-fusion proteins were incubated with the beads and then incubated with ^{35}S-labeled RXRα as described above

reciprocally, Z interferes with the binding of RXRα to its consensus binding site, a RARE sequence in the cellular promoter for RARβ (17). Here we test by affinity chromatography whether RARα and RXRα interact directly with GST-Z fusion protein. *In vitro*-translated ^{35}S-methionine-labelled RARα bound to beads coated with GST-Z (Fig. 1, lane 2), but not to the GST protein alone (lane 1) A mutant of Z, which retains only its DNA-binding and dimerization domain (ZΔ 1–86, lane 3), could still retain RARα whereas fusion proteins containing deletions in the coiled–coiled dimerization domain of Z (ZΔ 140–227 and ZΔ 200–227, lanes 4 and 5) could not. Similarly, labeled RXRα binds to GST-Z (lane 2) and to ZΔ 1–86 (lane 3) but not to the mutants of Z that have the dimerization domain deleted (lanes 4 and 5) as shown in Figure 1B. These results suggested that either RXRα and RARα interact within a region of Z corresponding to amino acids 140–227 or that homodimerization of Z is required for interaction with RXRα and RARα.

Z binds to RXRα in a region that is not required for homodimerization of Z

To define further the region of Z within the dimerization domain (Fig. 2A) that was required for interaction with RXRα, point mutants of Z (a generous gift from E. Flemington) were radiolabelled (as shown in Fig. 2B) and equal amounts of labeled protein used in affinity-chromatography experiments. These experiments led to the identification of mutants of Z that differentially bound to GST-Z and not to GST-RXRα. The wild-type Z protein bound well to GST-Z (Fig. 2C, lane 2) and GST-RXRα (lane 3) but not to GST alone (lane 1). Two mutants of Z (Z214R/218S and Z214S/218S) that cannot form Z homodimers in glutaraldehyde-crosslinking assays and did not bind to GST-Z (lanes 8 and 10), also did not bind to RXRα (lanes 9 and 11) suggesting that a dimer of Z may be required for this interaction. Both these mutations are at the interface of the predicted Z coiled–coiled homodimer. Another mutant (Z209R/216E), with mutations introduced in residues predicted to be on the back of the helix binds to GST-Z (lane 12) as expected and also interacts with GST-RXRα (lane 13). Furthermore, a protein with mutations of two alanine residues also predicted to be at the back of the helix (Z205R/206D, lane 6) could interact with Z. However, this mutant could not bind to RXRα (lane 7), identifying a region in Z not needed for homodimerization but likely to be involved in its interaction with RXRα. Surprisingly, two Z mutants predicted not to dimerize based on glutaraldehyde-crosslinking studies (26) (Z197K/200S and Z200E/225E) could still interact with GST-Z in the GST-affinity chromatography assays (lanes 4 and 14), but were unable to interact with GST-RXRα (lanes 5 and 15). The results suggest that preservation of a core Z homodimerization domain is needed for interaction with RXRα. However the subdomain of Z that governs the ability to homodimerize may be different from that required to bind to RXRα. Thus we have identified three mutants of Z that could interact with GST-Z but not with GST-RXRα.

DNA-binding by Z mutants

To determine if the Z point mutants could bind to a Z-DNA binding site, we used the *in vitro*-transcribed and translated proteins in electrophoretic mobility shift assays. A ^{32}P-labeled 17 bp oligonucleotide containing the AP1 site was incubated with wild-type or mutant Z proteins. As shown in Figure 3, wild-type Z binds to the AP1 site (lane 2), and addition of cold AP1 oligonucleotide competes for this binding (lane 1). Mutants (Z205R/206D, 200E/225E and Z209R/216E) that can still dimerize to Z bind to the AP1 site as well as the wild-type Z, whereas Z197K/200S, which also homodimerizes, does not bind to DNA as well. Mutants Z214R/218R and 214S/218S do not bind to DNA.

Mutants of Z that retain the transactivation function but not the repressor function

When a construct containing the RARβ promoter (BRE-CAT) is co-transfected with any of the retinoic acid receptors (RARα, γ and RXRα) in the presence of RA, there is an increase in the promoter activity. We have shown that when the same promoter is co-transfected with these RARs and Z, promoter activity is completely inhibited (17 and Fig. 4A) and that inhibition is due to a direct interaction of the two proteins which results in Z

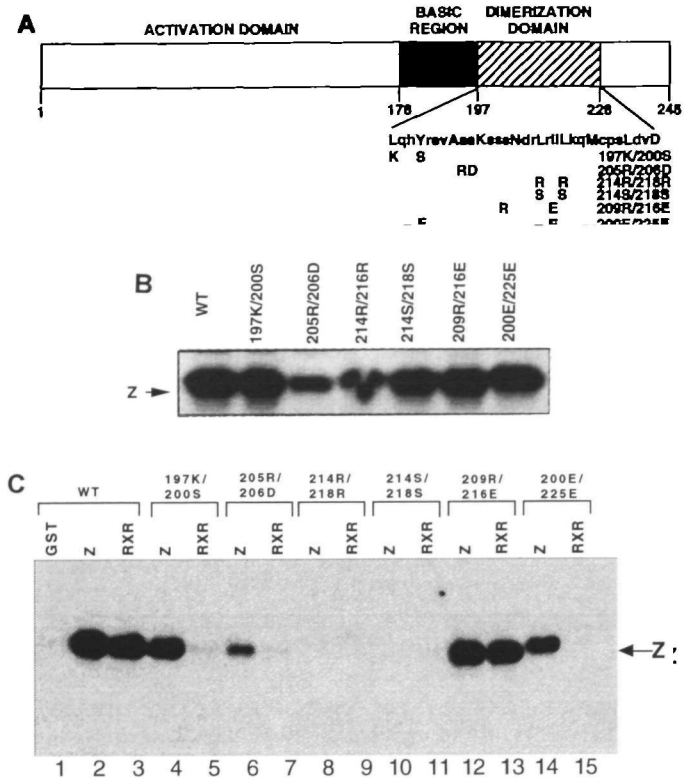

Figure 2. Interaction of Z mutants with RXRα. Protein binding experiments were done using Z mutants having point mutations within the dimerization domain of Z as described (**A**) Schematic diagram of the functional domains of Z and location of the mutations (**B**) Wild-type (lanes 1–3) or mutant Z proteins that homodimerize, Z197K/200S (lanes 4 and 5), Z205R/206D (lanes 6 and 7), 209E/216E (lanes 12 and 13) or 200E/225E (lanes 14 and 15) or mutants that did not homodimerize, Z214R/218R, Z214S/218S (lanes 8–11) were transcribed and translated *in vitro* using ^{35}S-methionine and analyzed on a 10% SDS/PAGE (**C**) Proteins were incubated with either GST alone, or GST-Z or GST-RXRα fusion proteins attached to GST–Sepharose beads, washed and boiled samples analyzed on a 10% SDS/PAGE.

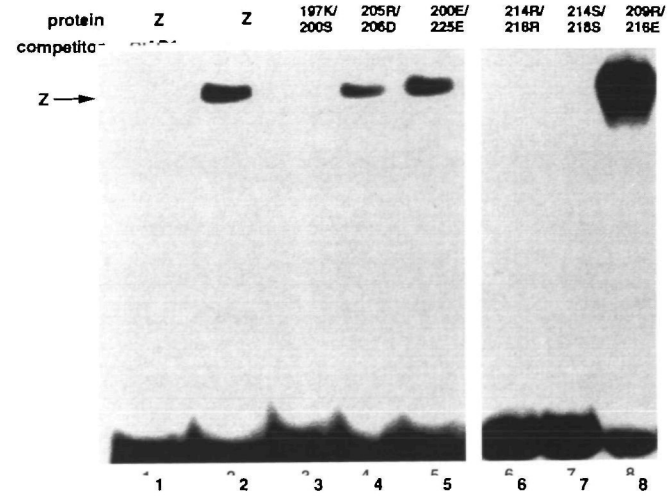

Figure 3. DNA-binding of Z mutants Wild-type (lanes 1 and 2) or mutant Z proteins Z197K/200S (lane 3), Z205R/206D (lane 4), or 200E/225E (lane 5), Z214R/218R (lane 6), 214S/218S (lane 7) and Z209R/216E (lane 8) were transcribed and translated *in vitro* and incubated with radiolabelled DNA probe containing an intact AP1 site in reaction buffer as described in Materials and Methods

interfering with the binding of RARα to its cognate site in the promoter. To complement these *in vitro* studies we tested whether mutants of Z that were unable to bind to RXRα *in vitro* would no longer interact with RXRα in the cell as indicated by inability to down-regulate an RXRα-responsive promoter. BRE-CAT was co-transfected with expression clones of RXRα and each of the Z mutants into EBV$^-$ lymphoblastoid cells (Louckes) and treated with RA (1 μM) 24 h before harvesting. Repression of RXRα-mediated activity by wild-type Z protein was considered to be 100% (Fig. 4B, bar 1). Z protein with a deletion in the transactivation domain of Z (ZΔ 25–29) or Z209R/216E can still repress >90% of the activity (bars 2 and 10). However, when mutants that did not bind to RXRα *in vitro* such as Z197K/200S (bar 5) were co-transfected with RXRα, only a 22% repression of activity resulted. Similarly, co-transfection of Z205R/206D (bar 6) and Z200E/225E (bar 7) with RXRα (neither mutant binds to RXRα) had less effect repressing only 30 and 18%, respectively, of promoter activity. Z mutants (Z214R/218S and Z214S/218S) (bars 8 and 9) that interacted with neither Z nor RXRα could not repress transactivation of BRE-CAT by RXRα. The plasmids ZΔ 140–227 (bar 3) and ZΔ 200–227 (bar 4) serve as negative controls, since the Z protein expressed from these constructs does not contain its nuclear localization signal. Thus the mutants of Z that could not bind to RXRα *in vitro* could not repress RXRα-mediated transactivation *in vivo*.

To test further whether the *in vitro* results correspond with interactions *in vivo* we used mutants that could homodimerize (Z197K/200S, Z205R/206D, Z200E/225E and Z209R/216E); these mutants can transactivate the Z-responsive EBV early promoter, BMRF1. As shown in Figure 5, co-transfection of the BMRF1-CAT construct with Z results in ~70-fold increase in activity (bar 1). Co-transfection of Z197K/200S (bar 2) can still induce at least 28-fold increase in activity, and Z205E/206D (bar 3), Z200E/225E (bar 6) and Z209R/216E (bar 7) can transactivate as well as and sometimes better than wild-type Z corroborating the *in vitro* results. In contrast, Z214R/218S and Z214S/218S (bars 4 and 5), which did not interact with wild-type GST-Z *in vitro*, could not transactivate BMRF1-CAT.

Mapping of the RXRα domain required for interaction with Z

Steroid hormone receptors including RARs/RXRs have a tripartite modular structure. The receptors have three identified domains: transactivation, DNA-binding and ligand-binding. The ligand-binding domain encompasses amino acids 240–462 at the C-terminus. Studies of dimerization with RXRα and interactions with other proteins suggested that the homodimerization domain of RXRα was in the C-terminus of the protein spanning amino acids 415–462 (27–31). We tested a series of C-terminal-truncated proteins that were expressed as described in Materials and Methods (Fig. 6A). As shown in Figure 6B, wild-type RXRα binds just as well to GST-Z (lane 2) as to GST-RXRα (lane 3). A mutant protein containing deletions of 60 amino acids in the C-terminus of RXRα also binds well to GST-Z (lane 4) and weakly to GST-RXRα (lane 5). RXRα with 92 amino acids deleted can be retained by GST-Z (lane 6) but weakly if at all by GST-RXRα (lane 7). RXRα mutants with 122 or more amino

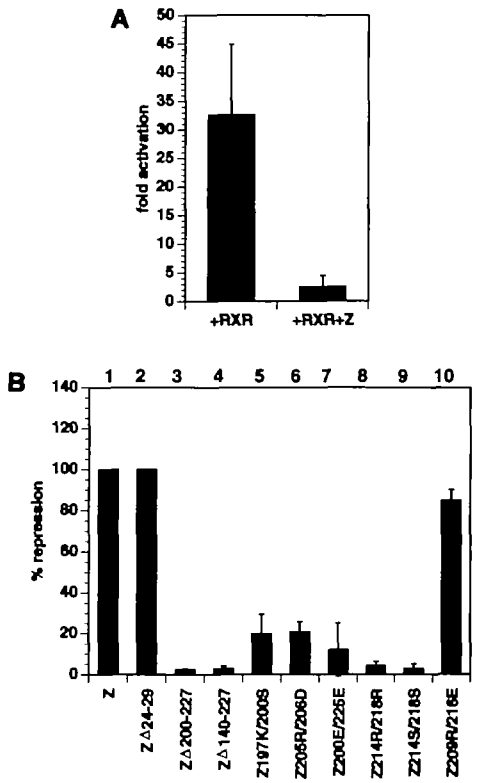

Figure 4. Effect of Z mutants on RXRα-mediated transactivation of the cellular promoter for RARβ. (**A**) The BRE-CAT construct was transfected into EBV-negative lymphoid cells, (Louckes) and co-transfected with pHD1013 (bar 1) or expression clones of wild-type Z protein (bar 2). (**B**) The BRE-CAT was also co-transfected with pHD1013, Z (bar1) or a protein with a mutation in the transactivation domain Z (ZΔ 25–29, bar 2), a protein with the leucine-zipper deleted (ZΔ 200–227, bar 3), a protein with 87 amino acids deleted from the dimerization domain of Z (ZΔ 140–227, bar 4) or point mutants that did not interact with RXRα, Z197K/200S (bar 5), Z205R/206D (bar 6), Z200E/225E (bar 7), Z214R/218R (bar 8), Z214S/218S (bar 9) and Z209R/216E (bar 10) as indicated. The cells were starved for 24 h before transfection, and RA (1 μM) was added 24 h after transfection. The promoter activity was assayed as described in Materials and Methods.

acids deleted from the C-terminus (lanes 8–15) had drastically reduced ability to bind to GST-Z or GST-RXRα. Therefore the domain of RXRα that interacts with Z seems to be separate from its homodimerization domain.

DISCUSSION

The EBV immediate-early protein, Z, is sufficient to trigger disruption of viral latency. Z, a member of the b-Zip family of proteins, forms homodimers and transactivates EBV early promoters containing *cis*-acting elements (ZREs) that start a cascade of events resulting in viral reactivation. Latency is almost certainly largely governed by cellular factors, and negation of leaky Z function would be necessary to maintain it. Retinoic acid receptors are excellent candidates for such regulatory cellular factors since they affect the status of cell differentiation which is important for different EBV infection states. Here we show that the reciprocal effects of retinoic acid receptors and Z in repressing activation of viral and cellular promoters are based on direct physical interaction between Z and RXRα. Mapping of the regions of Z and RXRα that are involved shows that RXRα binds

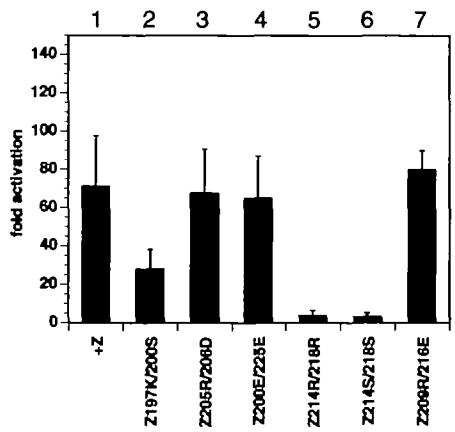

Figure 5. Effect of Z mutants on transactivation of the EBV BMRF1 promoter. The BMRF1-CAT construct was transfected into EBV-negative lymphoid cells (Louckes), and co-transfected with expression clones of wild-type Z protein (bar 1) or point mutants, Z197K/200S (bar 2), Z205R/206D (bar 3), Z200E/225E (bar 4), Z214R/218R (bar 5), Z214S/218S (bar 6) and Z209R/216E (bar 7). The cells were starved in charcoal-stripped serum for 24 h before transfection. Promoter activity was assayed as before

to Z within its dimerization domain (Fig. 1A). Furthermore, RARα also directly interacts with Z *in vitro* (Fig. 1B).

The Z protein functions as a coiled–coiled dimer (26). However, within this domain the region required for interaction with RXRα is distinct from that required for interaction with itself, as illustrated by the mutant Z205R/206D that can still bind to GST-Z but does not bind to RXRα (Fig. 2). The results differ from earlier results indicating that Z200E/225E could not form homodimers; here we show that this mutant can bind to GST-Z. Furthermore this mutant can bind to DNA (Fig. 3) and can transactivate a Z-responsive promoter (Fig. 5). The mutant DNA clones were confirmed by DNA sequencing. It is possible that these differences in the dimerization potential of the mutant Z proteins were detected because of the two different methods of analysis. Glutaraldehyde-crosslinking may not be as sensitive as GST-Z affinity chromatography. In our hands these mutants homodimerize well. It is also possible that the GST-Z fusion protein stabilizes the interaction with some of the mutants and thus binds to them under certain temperature and reaction conditions. However, the DNA-binding capacity of the mutants and the fact that these mutants can transactivate the BMRF1-CAT promoter construct in lymphoid cells as well as wild-type Z make a definitive case.

Point mutants of Z that differentially bind to Z but not to RXRα can be explained by the model shown in Figure 7. Z homodimers (or multimers) may present an interface for interaction with RXRα to form a higher order complex that is disrupted in Z mutants exemplified by Z205R/206D. Therefore these mutants can still homodimerize but can not bind to RXRα. Part of this interface may overlap with the dimerization interface, or there may be a change in conformation, so that mutants like Z197K/200S that dimerize well in this assay may not form dimers under some conditions. Mutants like Z209R/216E may be able to dimerize under both conditions, perhaps because the mutation is not in the interface presented to the receptor or the interface required for homodimerization. On the other hand, mutant Z214R/218R cannot homodimerize and therefore cannot present

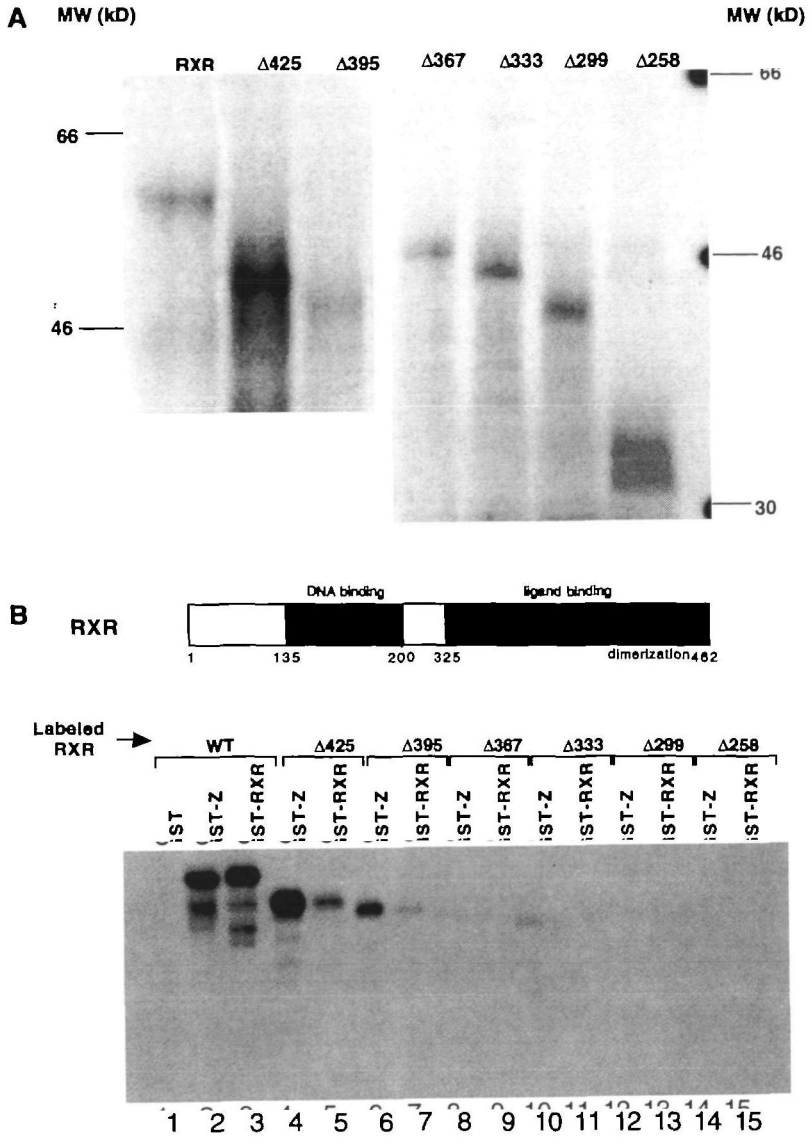

Figure 6. Interaction of RXRα mutants with Z. (**A**) The 3′ deletion mutants of RXRα were constructed by linearizing pBSRXRα with *Stu*I (1463), Δ425, (lanes 4 and 5) deleting 60 amino acids, *Bss*HII (1185), Δ395, deleting 92 amino acids, or with *Bam*HI (777), Δ258, deleting 257 amino acids Mutants spanning the region between *Bss*HII and *Bam*HI were obtained by PCR amplification as described in Material and Methods The PCR products were gel purified and used in *in vitro* transcription and translation using ^{35}S-methionine (**B**) Equal amounts of radiolabelled mutant RXRα proteins were incubated with GST-Z or GST-RXRα and analyzed using SDS/PAGE

an interface for interaction with dimers or multimers of RXRα to form a higher order complex.

The identification of Z mutants that differentially bind to Z and not RXRα enabled probing for effects of interaction between Z and RXRα in cells. Mutants of Z that did not bind to RXRα *in vitro* were incapable of repressing RXRα-mediated transactivation *in vivo* (Fig. 4). In contrast, these mutants could form homodimers and could transactivate the Z-responsive promoter as well as wild-type Z; Z mutants that did not homodimerize could not activate the promoter (Fig. 5). On the other hand, a mutant (Z197K/200S) which could not bind to DNA well could still transactivate the Z-responsive promoter, but transactivation was very variable and never as much as with wild-type Z. It has been reported that Z may have a transactivation function independent of DNA binding. Several groups have provided evidence that non-DNA binding forms of Z may be generated post-transcriptionally (32). Mutant Z proteins that cannot bind DNA have been shown to transactivate Z-responsive promoters. Transactivation by such Z mutants may be mediated through interaction with cellular transcription factors. Such DNA-binding mutants of Z can activate transcription through a cAMP-response element (CRE) and NF-κB sites but not through ZREs or AP1 (33). Perhaps Z197K/200S falls into this category of mutants and further characterization will be interesting.

Negative regulation by retinoic acid receptors is mediated through very different mechanisms, some of which involve a protein–protein interaction (20,21,34,35) Although RARα has been shown to inhibit DNA-binding of AP1, this is a demonstration of a physical interaction between a member of the steroid hormone family, which has a zinc-finger motif, and the leucine-

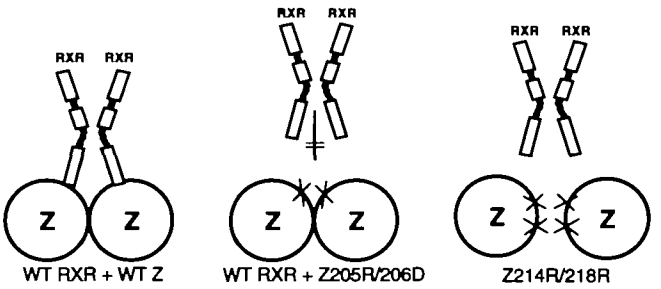

Figure 7. Physical interaction between RXRα and Z. A hypothetical model depicting the possible interactions between Z and RXRα. The wild-type Z dimers or multimers may present an interface for interaction with RXRα to form a higher order complex. In mutants like Z205R/206D, the interface may be disrupted although homodimerization of Z is still possible. In other mutants such as Z214R/218R, homodimers cannot be formed and thus do not allow the formation of the interface.

zipper-containing Z protein, which belongs to the b-Zip family. Mapping of the RXRα domain required for interaction has indicated that Z binds to an internal region of the ligand-binding domain of RXRα. The E region of the receptors is required for homodimerization of RXRα and heterodimerization with retinoic acid receptor (RAR) and thyroid hormone receptor (TR) (27–31). Forman and Samuels proposed that the ligand-binding domain encompasses nine short hydrophobic heptad regions (30). The ninth C-terminal heptad is required for the heterodimerization of RXRα with other members of the receptor family such as RAR and TR (36). In contrast, both vitamin D_3 receptor (VDR) and peroxisome-proliferator-activated receptor (PPAR) require additional N-terminal RXR sequences for heterodimerization (37). Although deletion of the C-terminal 40 amino acid segment from the ligand-binding domain of RXRα abolishes the interface for heterodimerization, homodimerization may require the region spanning amino acids 415–425. The DNA-binding domain also presents an interface for stable homodimer formation (38). The results indicate that the loss of the C-terminal 60 amino acids of RXRα, which deletes the ninth heptad but contains the DNA-binding domain, binds weakly to a GST-RXRα fusion protein as expected. However, loss of additional amino acids abolishes the ability of mutant RXRα to bind to GST-RXRα.

Interestingly, Z binds to RXRΔ401 (a mutant lacking the ninth heptad), indicating that the heterodimerization domain of RXRα may not be a target for Z interaction. Other members of the receptor family compete for the same interface of RXRα for heterodimerization, and therefore overexpression of one may result in repression of transactivation by another receptor. For example, since TR and RARα bind to the same interface of RXRα, overexpression of TR has been shown to repress RARα-mediated transactivation (36). These results suggest that Z does not compete with other members of the steroid hormone receptor family for binding to RXRα. Presumably repression of RXRα and RARα-mediated transactivation is due to direct interaction in a region different from that required for homo- or heterodimerization. Rather it is possible that Z dimers or multimers interact with RXRα homo- or heterodimers or multimers to form a higher order complex.

The potential to form homo- and heterodimers or higher order complexes among the members of the nuclear steroid hormone superfamily within the cell imparts increased diversity and versatility on the function of the nuclear receptors and thereby on nuclear hormones. Such combinatorial mechanisms also operate within families of transcription factors, including members of the leucine-zipper family and the c-*rel* oncogene family which allows fine tuning of regulation of gene expression (39,40). Intranuclear communication also occurs between members of two distinct families of transcription factors, adding another level of regulation. Such cross-talk has been detected between members of the AP1 family and nuclear receptors (22,35,41) or AP1 and the *rel* family (14,42) or, more recently, between the *rel* family and nuclear receptors (43). Viruses are dynamic participants in this intricate circuitry of signals in the cell. It is not surprising then that RARs transactivate the hepatitis B virus enhancer I (44) and initiate induction of the cytomegalovirus enhancer in embryonal cells (45). In contrast, RA has been shown to repress the expression of human papillomavirus 18 E6/E7 genes (46). The interaction between the EBV protein, Z, and RXRα provides an example of communication not only between members of different transcription factor families, but also between the viral and cell factors.

ACKNOWLEDGEMENTS

We thank Eric Flemington for very generous gifts of Z point mutants and Ronald Evans for the expression clones for RARα and RXRα as well as the antiserum for RXRα. We thank Alan Rickinson for the monoclonal antibody to Z, BZ-1. We are grateful to Shannon Kenney for Z fusion proteins and BMRF1-CAT and also for valuable discussions throughout the course of this work. This work was supported by grant # 2 PO1 CA19014-17A1 from the National Cancer Institute.

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