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Four EBNA2 Domains Are Important for EBNALP Coactivation

Chih-Wen Peng, Bo Zhao, and Elliott Kieff*

Department of Medicine and Microbiology and Molecular Genetics, Channing Laboratory, Brigham and Women's Hospital, and Harvard Medical School, Boston, Massachusetts

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EBNA2 transcriptional activation and regulated EBNALP coactivation are critical for Epstein-Barr virus-infected primary B-lymphocyte growth transformation. EBNALP coactivation requires the EBNA2 acidic activation domain (E2AD); EBNALP can bind to E2AD. EBNALP has now been found to bind less well to EBNA2 amino acids 1 to 58, which has been identified to be a second transcriptional activation domain, E2AD2. E2AD2 was specifically coactivated by EBNALP. Moreover, E2AD, E2AD2, EBNA2 RG domain, and the intermediate domain between RG and E2AD had significant roles in EBNA2-mediated activation and EBNALP coactivation.

Epstein-Barr virus (EBV) infection transforms resting B cells into permanent lymphoblastoid cell lines (for a review, see reference 14). In B lymphocytes, EBV nuclear antigen proteins, EBNA2 and EBNALP are the first two proteins expressed from the EBV genome. EBNA2 up-regulates transcription from specific cell and viral promoters through interactions with cell transcription factors that bind to cognate DNAs. EBNA2 amino acids (aa) 310 to 335 associate at a high level with RBPJk/CBF1, a key factor in Notch receptor gene activation. EBNA2 has a C-terminal acidic transcriptional activation domain (E2AD), which recruits basal and activation-related cell transcription factors and also binds EBNALP, enabling EBNALP to specifically coactivate transcription with EBNA2 (4, 10–12). In the context of Gal4 DNA binding domain (G4DBD) fusions, E2AD is a strong activator, which is repressed by EBNALP expression (12). Nevertheless, G4DBD-EBNA2 fusion proteins are coactivated by EBNALP, and coactivation by EBNALP is dependent on E2AD (4, 12). The objective of the experiments reported here is to identify the other components of EBNA2 that partially silence E2AD and enable EBNALP to coactivate.

EBNA2 aa 1 to 58 fused to G4DBD can activate Gal4-dependent transcription and is coactivated by EBNALP. To identify EBNA2 component(s) other than E2AD that may be coactivated by EBNALP, overlapping parts of the EBNA2 open reading frame were fused to G4DBD, which includes a nuclear localization sequence (2, 8), and were transfected into BJAB B lymphoblasts with a Gal4-dependent luciferase reporter in the presence or absence of an EBNALP expression vector (Fig. 1) (12). Plasmid CMV-βgal, a plasmid containing a cytomegalovirus (CMV) promoter and β-galactosidase reporter, was used as an internal transfection control. As previously described (12), G4DBD-EBNA2 aa 19 to 483 activated the Gal4-dependent promoter 10-fold, and coactivation with EBNALP resulted in additional 16-fold activation, resulting in 160-fold activation (Fig. 1). G4DBD-EBNA2 aa 1 to 30, 96 to 210, 200 to 334, and 300 to 432 failed to activate the Gal4-dependent promoter and were not coactivated by EBNALP (Fig. 1). Surprisingly, G4DBD-EBNA2 aa 1 to 58 activated transcription 5- to 10-fold, and coactivation with EBNALP resulted in additional 5- to 10-fold activation (Fig. 1). Thus, EBNA2 aa 1 to 58 is an activation domain that can be coactivated by EBNALP. Addition of the C-terminally adjacent polyproline (E2PP) domain, aa 59 to 95, suppressed activation, whereas EBNALP coactivation varied from 0- to 6-fold in multiple experiments (Fig. 1B, bars 3). We subsequently refer to EBNA2 aa 1 to 58 as a second EBNA2 activation domain, E2AD2. EBNA2 mutants in which aa 4 to 18 or 19 to 59 were deleted were previously noted to be deficient in activation of the latent membrane protein 1 promoter (LMP1), but not the Cp promoter (15).

E2AD activation and coactivation by EBNALP are affected by E2ID and E2RG sequences. In contrast to G4DBD-E2AD2, which has a 5- to 10-fold activating effect and is further coactivated 5- to 10-fold by EBNALP, G4DBD-E2AD aa 426 to 465, 433 to 465, and 433 to 483 activated transcription 100- to 3,000-fold and was ~30-fold repressed by EBNALP coexpression, as recently reported (12) (Fig. 2). N-terminal to E2AD, aa 426 to 465, is an E2 RG repeat domain, aa 335 to 362, which inhibits EBNA2 activation of LMP1 but not the Cp promoter, and an intermediate domain, E2ID, aa 363 to 425, which affects the activity of the RG domain (15, 17) (Fig. 1A and 2). Addition of only 21 E2ID residues to E2AD resulted in G4DBD-E2AD aa 405 to 465, which was five- to sixfold less active than G4DBD-E2AD and was not significantly repressed or activated by EBNALP coexpression (Fig. 2B). Addition of 15, 30, or 45 more E2ID residues yielded G4DBD-E2AD aa 390 to 465, 375 to 465, or 360 to 465, which lessened the repressive effect of the first N-terminal 21 aa but did not substantially alter the EBNALP nonresponsiveness that was seen with addition of the first N-terminal 21 aa to aa 426 to 465 (Fig. 2B). However, addition of the E2RG domain resulted in G4DBD-E2AD aa 335 to 465 or 335 to 483, which activated the Gal4-dependent promoter five- to sevenfold, and coactivation with EBNALP resulted in five- to sixfold activation for a total of 30-fold activation (Fig. 2B). Although the G4DBD-E2AD fusions varied in their expression levels, E2 activation

* Corresponding author. Mailing address: Department of Medicine and Microbiology and Molecular Genetics, Channing Laboratory, Brigham and Women's Hospital, and Harvard Medical School, 181 Longwood Ave., Boston, MA 02115. Phone: (617) 525-4252. Fax: (617) 525-4257. E-mail: ekieff@rics.bwh.harvard.edu.

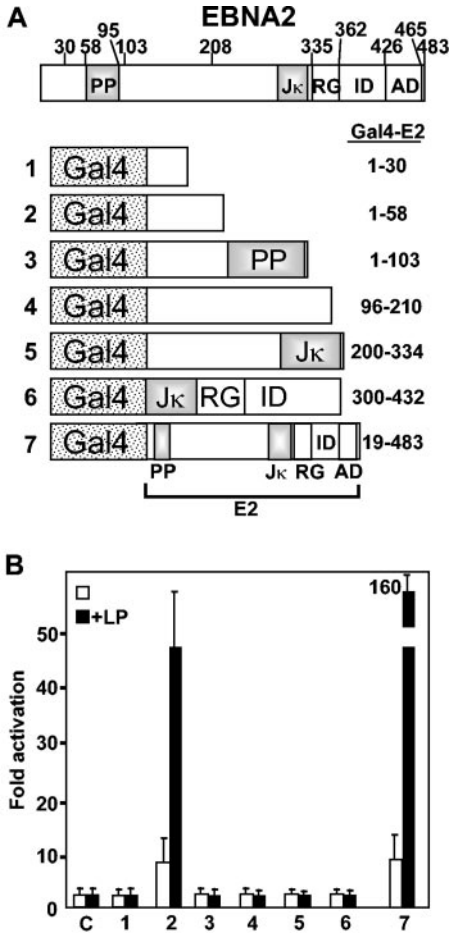

FIG. 1. Activation of a Gal4-dependent promoter and luciferase reporter by Gal4 DNA binding domain-EBNA2 (G4DBD-E2) fusion proteins and coactivation by EBNALP. (A) Schematic diagram of EBNA2 showing polyproline (PP) domain aa 59 to 95, core RBPJk (Jk) binding domain aa 310 to 334, RG domain aa 335 to 362, intermediate (ID) domain aa 363 to 425, and activation (AD) domain aa 426 to 465 (3, 6). (B) Overlapping parts of the EBNA2 open reading frame as shown in panel A were individually fused in frame to the G4DBD and assayed for activation of a Gal4-dependent promoter with a luciferase reporter (12). BJAB human lymphoblasts were transfected with 10 μg of a G4DBD-E2 fusion protein or a G4DBD control expression vector, 10 μg of pSG5 control expression vector, and 2 μg of CMV-βgal reporter plasmid as a transfection control. Fold activation over the level of the Gal4DBD control (C) is presented by the white bars. Other BJAB cells were transfected with the same plasmids in a similar manner, except for the addition of 10 μg of pSG5-EBNALP. Fold activation over the level of the Gal4DBD control by the G4DBD-E2 fusion proteins and EBNALP is shown by the black bars. Data shown in figures are from a single experiment, whereas bars indicate 1 standard deviation from at least three independent experiments.

and EBNALP coactivation did not directly correlate with levels of expression (Fig. 2B, compare the robust activation signals for bars 5, 7, and 8 with the low-level expression in the immunoblot shown below the activation data). These data indicate that E2AD coactivation by EBNALP is dependent on E2AD and at least two other domains: E2ID, which inhibits E2AD-mediated activation and minimizes the repressive effects of EBNALP, and E2RG, which strongly suppresses E2AD activation and aids EBNALP coactivation.

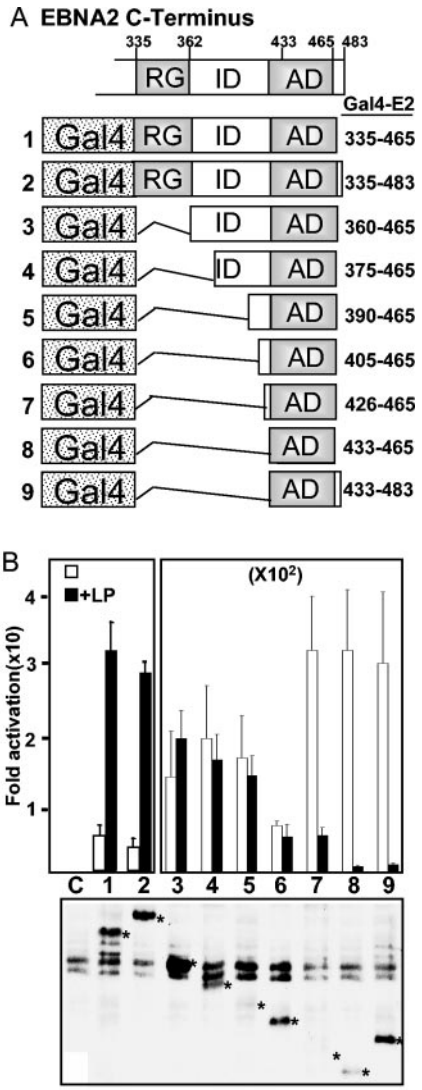

FIG. 2. Effects of additional EBNA2 amino acids that are N terminal to E2AD on G4DBD-E2AD activation of a Gal4-dependent promoter and luciferase reporter. The G4DBD-E2 fusion proteins used are schematically depicted in panel A. In panel B, the white bars labeled 1 to 9 show the fold activation by each G4DBD-E2 fusion protein shown in panel A relative to that of the G4DBD control (C). The fold coactivation by each G4DBD-E2 fusion protein and EBNALP relative to the G4DBD control (C) is shown by the black bars. The amounts of transfected DNAs were as specified in the legend to Fig. 1. Note that the fold activation panel is split with bars C, 1, and 2 having fold activation values of 1×10 to 4×10 as indicated in the ordinate description in the figure, while lanes 3 to 9 have 100-fold-higher activation dimensions of 1×10^3 to 4×10^3 (as indicated by the $\times 10^2$ in that panel). The relative expression levels of G4DBD-E2 fusion proteins by immunoblotting after transfection of BJAB cells with 20 μg of G4DBD fusion protein expression vector are shown in the blot below the graph. The fusion proteins were detected by using the PE2 E2AD-specific monoclonal antibody. Each Gal4-E2 fusion protein is marked with an asterisk.

E2AD, E2AD2, E2 RG, and the EBNA2 intermediate domain affect EBV Cp regulation. The roles of EBNA2 domains in activation and EBNALP coactivation of the Cp promoter (9) were evaluated using transient-transfection assays in BJAB

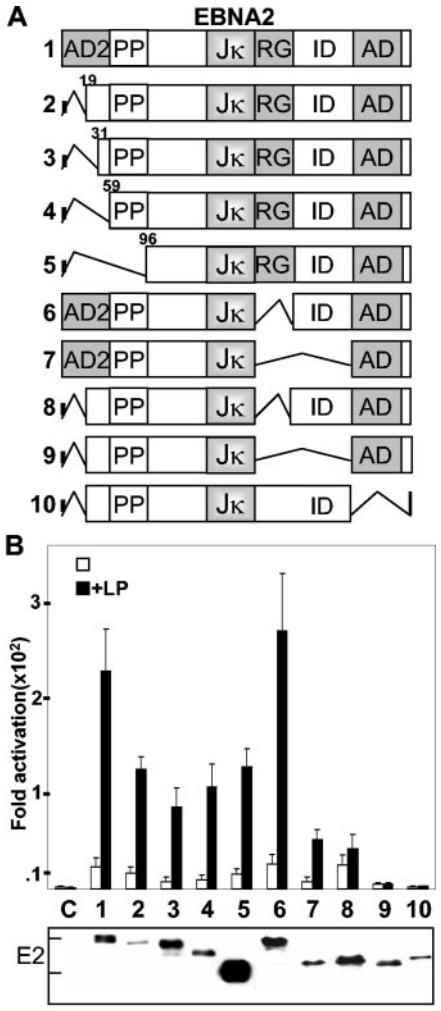

FIG. 3. EBNALP coactivation of the EBV Cp promoter with EBNA2 requires E2AD, E2AD2, E2RG, and E2ID. The wild-type or mutant EBNA2 proteins that were assayed for activation of the EBV Cp promoter and for coactivation of the Cp promoter by EBNALP are depicted schematically in panel A. Panel B shows the results of BJAB cell transfection with 5 μg of EBV Cp-Luc (12), 10 μg of the indicated wild-type or mutant EBNA2 expression plasmid or control expression vector, 10 μg of pSG5 vector control, and 2 μg of CMV β-gal transfection control reporter plasmid. Fold activation by wild-type or mutant EBNA2 relative to control expression vector (C) is indicated by the white bars. Fold coactivation by 10 μg of pSG5-EBNALP expression vector is shown by the black bars. Wild-type or mutant EBNA2 protein levels are shown in the immunoblot below the graph.

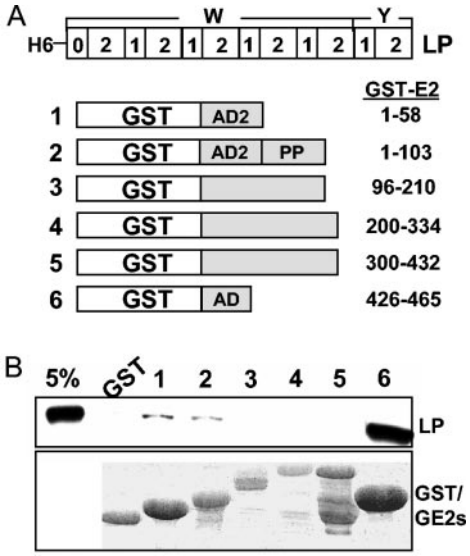

FIG. 4. Binding of His-tagged EBNALP purified from *Escherichia coli* (12) to GST fusion proteins with parts of the EBNA2 open reading frame. The EBNA2 codons that were fused in frame to the 3′ end of the GST open reading frame (12) are shown in panel A. GST fusion proteins on beads were incubated with His-tagged EBNALP and washed extensively with binding buffer. Bound His-tagged EBNALP was assayed by immunoblotting with JF186 monoclonal antibody to EBNALP, relative to 5% of the input His-tagged EBNALP for each binding assay mixture (12) in panel B. The amount of each fusion protein was assayed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie brilliant blue staining in the bottom gel and estimated relative to control bovine serum albumin staining as 5 μg of GST, 10 μg of GST-E2 aa 1 to 58, 5 μg of GST-E2 aa 1 to 103, 3 μg of GST-E2 aa 96 to 210, 2 μg of GST-E2 aa 200 to 334, 5 μg of GST-E2 aa 300 to 432, and 20 μg of GST-E2 aa 426 to 465 for lanes GST to 6, respectively.

lymphoblasts (Fig. 3). As expected (12), EBNA2 activated Cp transcription ~10-fold, and coactivation with EBNALP further increased transcription ~20-fold, resulting in a total activation of 200-fold (Fig. 3B, bars 1). Progressive deletion of E2AD2 or of E2AD2 and E2PP reduced EBNA2 activation two- to threefold in several experiments but did not substantially affect coactivation with EBNALP, which resulted in 100-fold total activation (Fig. 3B, bars 2 to 5). Deletion of E2RG or of E2RG and E2ID had a slight positive effect or at most a four- to fivefold overall negative effect on EBNA2 activation as previously noted (15, 17) and had no effect or a four- to fivefold effect on coactivation with EBNALP, with ~270- or ~40-fold total coactivation, respectively (Fig. 3B, bars 6 or 7, respectively). In contrast, deletion of E2AD2 aa 2 to 19 and E2RG had almost no effect on EBNA2 activation, whereas EBNALP coactivation was substantially decreased (Fig. 3B, bars 8). More extensive deletion of E2AD2 aa 2 to 19, E2RG, and E2ID resulted in complete loss of EBNA2 activation and EBNALP coactivation, despite detectable EBNA2 expression (Fig. 3B, lanes 8 and 9). The role of E2AD2 aa 2 to 19 in enabling EBNALP coactivation in the absence of the RG domain is most obvious in Fig. 3 (compare lane 6 with lane 8 and lane 7 with lane 9). These data indicate that E2AD2, E2RG, and E2ID can have important roles in EBNA2 activation and EBNALP coactivation.

EBNALP binds primarily to E2AD, but it also specifically binds to E2AD2. To investigate the basis for the specific coactivating effect of EBNALP with E2AD2, we evaluated the binding of bacterially expressed His_6-tagged EBNALP (12) to bacterially expressed glutathione *S*-transferase (GST) fusions with overlapping parts of the EBNA2 open reading frame. EBNALP specifically bound to GST-E2AD at a high level as reported previously (12) and also bound to GST-E2AD2 aa 1 to 58 and 1 to 103 at a low level (Fig. 4B). EBNALP did not bind to GST-EBNA2 (aa 96 to 210, 200 to 234, or 300 to 432) or to GST despite equivalent amounts of these other proteins (Fig. 4B). Thus, direct EBNALP interaction with E2AD and

E2AD2 may be an important basis for specific coactivation, although high-level stable association is incompatible with EBNALP coactivation (12).

E2AD is a strong transcriptional activator, particularly in B lymphocytes, and is essential for EBNA2 activation of cell and viral gene transcription and for EBNALP coactivation (1–3, 12). EBNALP expression at any level represses the strong constitutive activity of E2AD fused to G4DBD (12). The data presented here identify EBNA2 domains that down-regulate the high-level intrinsic E2AD activity and enable EBNALP to coactivate with EBNA2. The effects described here were reproducible in at least three independent experiments. The effects differ to a limited extent in the two assays that were used and will likely vary more in assays using other EBNA2-responsive promoters or other cell types.

First, E2AD2 corresponds to a self-associating EBNA2 domain (5), which in this study was found to convey independent activation when fused to G4DBD and was then coactivated by EBNALP. Deletion of even part of this domain resulted in less EBNA2 activation and EBNALP coactivation of the Cp promoter and left E2AD highly dependent on the RG and intermediate domain for EBNA2 activation and EBNALP coactivation of the Cp promoter (Fig. 3). These data define new roles for the EBNA2 domain from aa 1 to 58 as a transcriptional activator, which can be coactivated by EBNALP, and implicate this domain in interactions with a transcriptional activator, which remains to be identified.

Second, the EBNA2 RG domain, E2RG, is a protein-protein and protein-nucleic acid interaction domain, important for efficient cell growth transformation, and a down-regulator of EBNA2 activation of the LMP1 promoter, but not the Cp promoter (15, 17). The E2RG domain was an important repressor of G4DBD-E2AD-mediated promoter activation and enabled EBNALP coactivation; the RG domain was particularly critical for EBNALP coactivation of the Cp promoter when aa 1 to 19 in EBNA2 had been deleted (Fig. 3). Given the critical role of EBNALP charged residues and phosphorylation in interactions with E2AD and in EBNA2 coactivation by EBNALP, we speculate that the E2RG domain may have a similar role in intra- and possibly intermolecular effects on EBNA2 activation and EBNALP coactivation (7, 12, 13, 16).

Third, although the EBNA2 intermediate domain, E2ID, is nonessential for B-cell growth transformation (17), E2ID significantly affected E2AD-mediated activation and the effects of EBNALP on E2AD. E2ID down-regulated the very high G4DBD-E2AD activity; G4DBD-E2IDAD was less active than G4DBD-E2AD (Fig. 2). Further, EBNALP coexpression with G4DBD-E2IDAD did not inhibit G4DBD-E2IDAD-mediated activation, as was evident with G4DBD-E2AD (Fig. 2). Addition of only 20 aa of ID (EBNA2 aa 405 to 425) to G4DBD-E2AD inhibited E2AD activation and down-regulated the repressive effect of EBNALP. In assays of EBNA2 activation of the Cp promoter, E2ID had a positive effect on EBNA2 activation and EBNALP coactivation, as evident in Fig. 3.

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