Multivalent Sequence Recognition by Epstein-Barr Virus Zta Requires Cysteine 171 and an Extension of the Canonical B-ZIP Domain

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Epstein-Barr virus (EBV) immediate-early protein Zta is a member of the basic-leucine zipper (B-ZIP) family of DNA binding proteins that has an unusual capacity to recognize multiple DNA recognition sites, including AP-1 and C/EBP binding sites. To better understand the structure and function of Zta, we have mutagenized cysteine residues within or adjacent to the B-ZIP domain. We found that serine substitution for cysteine 171 (C171S), which lies outside and amino terminal to the B-ZIP basic region, completely abrogates Zta capacity to initiate lytic cycle replication. C171S disrupted Zta transcription activation function of several EBV lytic cycle promoters, including the BMRF1 gene (EA-D) and the other lytic activator, Rta. Overexpression of Rta could not rescue the C171S defect for transcription reactivation or viral DNA replication. Zta C171S was defective for binding to these promoters in vivo, as measured by chromatin immunoprecipitation assay. Purified Zta C171S bound AP-1 sites similar to wild-type Zta, but it was incapable of binding several degenerate Zta sites, including a consensus C/EBP site. Zta truncation mutations reveal that residues N terminal to the B-ZIP (amino acids 156 to 178) confer C/EBP binding capacity to the otherwise AP-1-restricted DNA recognition function. Comparison among viral orthologues of Zta suggest that a conserved N-terminal extension of the consensus B-ZIP domain is required for this multivalent DNA recognition capacity of Zta and is essential for viral reactivation.

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bution of these critical residues to Zta structure and function remain elusive. In the studies presented here, we further explore the role of cysteine residues in Zta function and present evidence that a second cysteine at position 171 (C171) is essential for reactivation and for sequence recognition of C/EBP-like sites. These findings suggest that amino acid residues amino terminal to the consensus B-ZIP domain contribute to sequence recognition by a mechanism not revealed in the crystal structure, but they are nevertheless essential for lytic reactivation.

MATERIALS AND METHODS

Point mutations in Zta were generated by PCR mutagenesis using the Quick-change site-directed mutagenesis kit (Stratagene). Full-length Zta proteins with cysteine substitution mutations were cloned into the BamHI site of a pQE8 bacterial expression vector (QIAGEN). Zta truncation mutations N156 and N166 were cloned into the NheI-HindIII sites of pET28a. Zta N178 was cloned into the BamHI site of pQE8. Zta wild type (wt), C171S, and C132S were cloned as EcoRI-SalI fragments in p3XFLAG-myc-CMV24 vector (Sigma) for mammalian cell expression. Luciferase plasmids for Zp, Rp, Hp, and Mp have been described previously (14). AP-1/SV-LUC and C/EBP/SV-LUC were generated by cloning the AP-1 oligonucleotide (5’GTACCACTGACTCATCACTGACTCATG) or the C/EBP oligonucleotide (5’GTACATTGCGCAATCATTGCGCAATG) into the Asp1/Nhe1 site of a pGL2 promoter (Invitrogen) which contains the simian virus 40 early promoter region upstream of the luciferase gene. Rta expression vector pRTS15 was a gift from S. D. Hayward and contains the BRLF1 open reading frame in a derivative of pSG5 (Stratagene). All of the methods for this study have been described previously (35). Sequences of oligonucleotide probes for electrophoretic mobility shift assay (EMSA) have been published previously (31, 35).

RESULTS AND DISCUSSION

Identification of cysteine residues essential for lytic cycle replication. A series of cysteine-to-serine mutations were introduced into Zta (Fig. 1A). Previous studies demonstrated that combinations of these cysteines were responsible for redox-sensitive DNA binding but that no single cysteine could completely account for this activity (35). To further explore the molecular biology of these cysteine residues in mediating Zta functions, we assayed single cysteine-to-serine mutations at positions 189 and 222 as well as combined mutations at positions 189 and 222 (designated 189/222), 132/189/222, 171/189/222, and 132/171/189/222. These cysteine substitution mutations were expressed as FLAG-tagged proteins in ZKO-293 cells to determine their ability to stimulate viral reactivation and lytic gene expression.
tivated EA-D and Rta in a manner indistinguishable from that of wild-type Zta (Fig. 1B). In contrast, C171/189/222S and C132/171/189/222S were significantly reduced for EA-D and Rta transcription activation (Fig. 1B). These Zta mutants were then tested for their ability to stimulate production of infectious virus (Fig. 1C) and amplification of EBV genome DNA (Fig. 1D). We found the C189S was defective for production of infectious virus and DNA amplification, consistent with our previous report that this cysteine residue was critical for viral lytic cycle replication (35). We also observed that C171/189/222S and C132/171/189/222S were reduced for production of infectious virus (Fig. 1C) and completely defective for amplification of genomic DNA (Fig. 1D). These findings indicate that combinations of mutations containing C171S were highly defective for transcription activation of some viral early genes and were incapable of lytic cycle DNA replication.

**Cysteine 171 is required for viral reactivation.** To determine if single-serine substitution mutations at C132 or C171 reduced Zta activity, we expressed C132S and C171S in ZKO-293 cells (Fig. 2A) and assayed by Western blot for activation of early lytic antigens EA-D and Rta. We found that C132S behaved essentially like wild-type Zta, while C171S was defective for activation of EA-D and Rta (Fig. 2A). This defect of C171S was further validated by measuring the mRNA levels by reverse transcription-PCR (Fig. 2B). C171S was incapable of stimulating EA-D or Rta mRNA levels despite identical levels of Zta and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control mRNA in each reaction. Expression levels for Zta wt, C132S, and C171S were nearly identical, indicating that these differences in transcription activity were not attributable to differences in protein expression levels.

These findings indicate that C171S is defective for transcription activation of all of the viral early promoters tested but may also have more pronounced defects at some subset of these promoters.

We next determined whether Zta C171S was defective for binding to the target promoter regions of EBV DNA in vivo using real-time PCR analysis of chromatin-immunoprecipitated (ChIP) DNA (Fig. 4A to D). We found that C171S was substantially reduced for binding at all promoters tested relative to wt Zta. Binding at BHRF1p and BFRF1p was reduced approximately six- to sevenfold relative to wild-type Zta (Fig. 4A and B), while binding at BMRF1p and BRLF1p was reduced only approximately twofold relative to wt Zta (Fig. 4C and D). These findings suggest that C171S impairs Zta DNA binding capabilities but has more penetrating effects on the DNA binding at some sites (e.g., BHRF1) relative to other sites (BMRF1p).

Since reactivation is strongly dependent on Rta coexpres-
sion, and since C171S is defective for transcription activation of Rta through the BRLF1 promoter, we tested whether overexpression of Rta could rescue the C171S defect. Others have found that Rta could rescue the reactivation defects in other Zta mutations, especially the S186A mutation that is defective for binding methylated DNA at the BRLF1 promoter (2). An Rta expression vector was cotransfected with or without Zta wt, C132S, or C171S expression vectors and assayed for Zta, Rta, and EA-D expression (Fig. 4E), as well as for viral DNA amplification (Fig. 4F) in ZKO-293 cells. Short exposures of Western blots revealed that EA-D levels were elevated by cotransfection of Rta with Zta wt and Zta C131S but not significantly in the presence of Zta C171S (Fig. 4E, middle panel). Longer exposure times indicate that Rta coexpression does stimulate EA-D expression in the presence of C171S but to a lesser extent than that of C131S or wt Zta (Fig. 4E, top panel). We also noted that Rta levels were consistently lower in the presence of Zta C171S, suggesting that stabilizing interactions between Zta and Rta may also be affected by mutations in Zta. Expression levels of Zta were nearly identical for wt, C132S, and C171S, as were control levels for cellular PCNA. We next asked whether the addition of Rta was sufficient to rescue the defect in C171S in lytic DNA replication (Fig. 4F). We found that Rta cotransfection had a slight stimulation on the DNA replication of C132S but had no detectable effect on C171S, which was indistinguishable from vector control, while Zta wt stimulated viral DNA ~70-fold. We conclude from these studies that Rta cotransfection cannot rescue the defect of C171S on lytic DNA replication or EA-D transcription activation. These findings indicate that the defect in Zta C171S is not limited to the expression of Rta through the transcription activation of the BRLF1 promoter.

C171 is required for binding to C/EBP sites but not AP-1 sites. The transcription activation defects at several different promoters (Fig. 3) and the failure of Zta C171S to bind BHLF1 and BHRF1 promoter regions by ChIP assay (Fig. 4A to D) suggest that C171S has a primary defect in DNA binding at some, but not necessarily all, recognition sites. Therefore, we assayed the DNA binding activity of purified Zta proteins

FIG. 3. C171S is defective for transcription activation of viral promoters. 293 cells were transfected with vector, Zta wt, C132S, or C171S and assayed for activation of luciferase reporter plasmids for EBV promoters BMRFL1(Mp)-Luc (A), BHLF1(Hp)-Luc (B), BRLF1(Rp)-Luc (C), or BZLF1(Zp)-Luc (D). (E) A Western blot of Zta proteins after a typical transfection assay.

FIG. 4. C171S is defective for binding to viral promoters in vivo. (A to D) Chromatin immunoprecipitation assays were performed with antibody to Zta or control immunoglobulin G (IgG) after transfection of ZKO-293 cells with vector, wt Zta, or C171S. ChIP DNA was assayed at BHLF1p (A), BHRF1p (B), BMRFL1p (C), or BRLF1p (D) and quantitated by real-time PCR for DNA recovered by Zta-specific antibody relative to the IgG control. (E) Western blot analysis of ZKO-293 cells transfected with (+) or without (−) Rta expression vector and either vector, Zta wt, Zta C132S, or Zta C171S. Long exposure times for chemiluminescence development are indicated for detection with Rta- and EA-D-specific antibodies (top panel), and short exposures are shown for Rta, EA-D, and Zta (middle panel). Levels of control cellular protein PCNA are indicated in the lower panel. (F) Viral lytic replication was measured by real-time PCR of viral DNA relative to cellular actin DNA in ZKO-293 cells transfected with Zta wt, C132S, or C171S cotransfected with (black) or without (gray) Rta expression plasmid.
for several different Zta recognition sites in vitro using electrophoretic mobility shift assay (EMSA). C171S, C132S, and wt Zta were expressed and purified from *Escherichia coli* and adjusted to identical protein concentrations (Fig. 5A). These proteins were assayed for DNA binding to several Zta recognition sequences. We found that C171S bound the BRLF1 ZRE2 site with affinity nearly identical to that of wild-type Zta and C132S (Fig. 5B). In contrast, Zta C171S was highly defective for binding the more degenerate recognition site ZRE3, also derived from the BRLF1 promoter, relative to Zta wt or C132S (Fig. 5C). A similar defect in Zta C171S was observed when tested for binding to the cytosine-methylated form of these ZRE3 sites (Fig. 5D), with no significant change in wt or C132S Zta. These findings suggest that C171S limits the variety of sites recognized by Zta. To further explore this possibility, we compared C171S with wt and C132S Zta for binding to 11-bp probes that contain only the AP-1 or C/EBP recognition sites as described in the crystallographic studies (31). We found that Zta C171S bound the AP-1 site at levels similar to those of Zta wt and C132S (Fig. 5E). In striking contrast, C171S was incapable of binding the C/EBP site probe, while the wt and C132S Zta proteins bound with similar affinity to that of the AP-1 site (Fig. 5F). These findings indicate that C171S abrogates the C/EBP site recognition capability of Zta but has limited effect on the AP-1 binding activity.

To determine if this difference in DNA binding recognition correlated with transcription activation function, we generated reporter constructs with either 3× AP-1 sites or 3× C/EBP sites in the pGL2 promoter construct, which places these test sequences immediately upstream of the simian virus early promoter driving the luciferase gene (Fig. 6A). We compared wt and C171S Zta expression vectors for their ability to stimulate transcription from either 3× AP-1/SV-LUC or 3× C/EBP/SV-LUC in 293 cells. We found that Zta C171S stimulated the AP-1-containing construct to levels ~48% of that of wt Zta (Fig. 6B and C). In contrast, C171S stimulated the C/EBP-containing construct to only ~13% of that of wt Zta (Fig. 6B and C). These findings indicate that C171S was approximately threefold more defective for transcription activation of the C/EBP-containing promoter than the AP-1-containing promoter. This activation correlates well with the difference in DNA binding activity (Fig. 5E and F) and further supports our model that C171S affects the DNA recognition capabilities of Zta.

Evidence for a conserved amino-terminal extension of the B-ZIP basic region required for multiple-sequence recognition. A comparison of Zta proteins from the related cercopithecine herpesvirus 15 and callitrichine herpesvirus 3 revealed a strong conservation of the C171 residue as well as an
additional stretch of residues extending to amino acid 156 of Zta (Fig. 7A). To test if this additional stretch of conserved amino acids contribute to DNA binding recognition, we compared Zta proteins with N termini initiating at amino acid 156 (N156), 166 (N166), or 178 (N178) (Fig. 7B). These proteins were expressed and purified from E. coli and adjusted for protein concentration by Coomassie staining (Fig. 7C), and they were then assayed for their ability to bind to the AP-1 or C/EBP 11-mer probes (Fig. 7D). We found that the shortest derivative, N178, bound to AP-1 weakly and did not bind to the C/EBP probe. The N166 truncation bound to the AP-1 probe with high affinity (lanes 5 to 7) but did not bind detectably to the C/EBP probe (lanes 15 to 17). In contrast, the N156 truncation bound with high affinity to AP-1 (lanes 8 to 10) and nearly similar affinity to the C/EBP probe (lanes 18 to 20). These findings indicate that the amino acids N terminal to the canonical basic region of the B-ZIP domain stimulate binding to a C/EBP recognition site. Since the N156 truncation did not bind C/EBP with the same affinity as the AP-1 site but the full-length protein bound both sites with equal affinity (Fig. 5E...
and F), it is likely that additional N-terminal amino acids contribute further to the recognition of C/EBP and other non-AP-1 consensus sites found throughout the viral and cellular genome.

Conclusions. In this work, we found that serine substitution for cysteine 171 disrupted the transcription activation and lytic cycle replication function of Zta (Fig. 2). C171S was defective for binding to viral target sequences at several viral promoters in vivo using ChIP assay (Fig. A4 to D), and purified recombinant C171S was defective for binding to a subset of target sites in vitro (Fig. 5). C171S in the context of full-length Zta protein bound to AP-1-like sites with nearly equal affinity to wt Zta but did not bind to several non-AP-1 consensus sites or to a consensus C/EBP site. Truncation mutations revealed that amino acids N terminal to the B-ZIP homology domain contribute to the C/EBP site recognition more significantly than to the AP-1 site. X-ray crystallography studies with a Zta truncation protein starting at amino acid 175 showed detectable but reduced binding to C/EBP sites relative to AP-1 sites (31). These structural studies are essentially consistent with our findings, although we now propose that amino acids N terminal to the basic region contribute significantly to multiple-sequence recognition. Additional structural studies with Zta peptides extended in the amino-terminal direction may be informative and may reveal a novel stabilization domain similar to that observed at the C-terminal side of the zipper/dimerization domain.

In addition to these structural implications, our data also indicate that multiple-sequence recognition by Zta is required for lytic cycle reactivation. While we cannot rule out that C171S eliminates some essential protein-protein interactions as well, our data are most consistent with the interpretation that multiple-site recognition properties of Zta are essential for completion of lytic cycle gene expression and DNA replication. Finally, it should be noted that regions amino terminal to other basic homology regions have been described, including the “cap n collar” motif of the MAF family of proteins, which contribute to DNA recognition during the oxidative stress response (30). We suspect that the region N terminal to the basic homology domain of Zta represents a conserved motif among Zta family members that is essential for multivalent DNA site recognition and other interactions required for lytic cycle activation.

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