A Redox-Sensitive Cysteine in Zta Is Required for Epstein-Barr Virus Lytic Cycle DNA Replication

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Epstein-Barr virus (EBV) reactivation from latency is known to be sensitive to redox regulation. The immediate-early protein Zta is a member of the basic-leucine zipper (bZIP) family of DNA binding proteins that stimulates viral and cellular transcription and nucleates a replication complex at the viral lytic origin. Zta shares with several members of the bZIP family a conserved cysteine residue (C189) that confers redox regulation of DNA binding. In this work, we show that replacement of C189 with serine (C189S) eliminated lytic cycle DNA replication function of Zta. The mechanistic basis for this replication defect was investigated. We show that C189S was not significantly altered for DNA binding activity in vitro or in vivo. We also show that C189S was not defective for transcription activation of EBV early gene promoters. C189S was deficient in binding methylated DNA binding sites and was capable of activating Rta from endogenous latent viral genomes, in contrast to the previously characterized S186A mutation. C189S was slightly impaired for its ability to form a stable complex with Rta, although this did not prevent Rta recruitment to OriLyt. C189S did provide some resistance to oxidation and nitrosylation, which potently inhibit Zta DNA binding activity in vitro. Interestingly, this redox sensitivity was not strictly dependent on C189S but involved additional cysteine residues in Zta. These results provide evidence that the conserved cysteine in the bZIP domain of Zta plays a primary role in EBV lytic cycle DNA replication.

Epstein-Barr virus (EBV) is a human gammaherpesvirus associated with endemic Burkitt’s lymphoma, nasopharyngeal carcinoma, and lymphoproliferative disorders in the immunosuppressed (39, 58). Like all herpesviruses, EBV can switch from latent to lytic infection through complex changes in gene expression and replication mechanisms. EBV can be cultured as a latent infection in immortalized B lymphocytes and Burkitt lymphoma-derived cell lines. During latency, EBV persists as a multicopy episome that expresses a limited subset of viral genes required for viral and cellular maintenance (66, 67). The switch to lytic cycle gene expression and DNA replication, referred to as reactivation, may occur spontaneously or may be initiated by various cellular signaling pathways, including B-cell receptor ligation and plasma cell differentiation (41, 64, 69). In vitro, latently infected B cells can be stimulated to undergo lytic replication by several chemical manipulations, including calcium ionophores (23), phorbol esters (56), halogenated nucleotides (65), and histone deacetylase inhibitors (48). Virus production can be detected in most EBV-positive adults and is thought to account for the high prevalence of EBV infection in the human population (35). Chronic lytic cycle gene expression is associated with oral hairy leukoplakia in AIDS patients (33, 42) and increased risk of nasopharyngeal carcinoma in regions where the virus is endemic (19).

Lytic replication and gene expression can be initiated by activation of the viral immediate-early protein Zta (also referred to as BZLF1, ZEBRA, and EB1) (17, 18). Zta is a member of the basic leucine zipper (bZIP) family of DNA binding proteins with sequence similarity to C/EBP, c-Jun, and c-Fos (40). Zta binds multiple recognition sites, including AP1 sites, and activates transcription of both viral and cellular genes (14, 37, 45, 46). One important viral gene target of Zta is Rta, a second immediate-early gene that can be coordinately expressed as a bicistronic RNA transcript with Zta (49). Rta and Zta function synergistically at some promoters and are both required for the completion of the viral lytic cycle (24). Rta rather than Zta is more highly conserved among gamma-herpesviruses and is the predominant lytic activator in the related rhadinoviruses Kaposi’s sarcoma-associated herpesvirus (KSHV)/human herpesvirus type 8 and HVS (32, 68, 84). Zta binds directly to the EBV origin of lytic replication and recruits the virus-encoded DNA primase and polymerase processivity factors that are essential for DNA replication (31, 46, 62). Viruses lacking Zta are incapable of lytic cycle gene expression or DNA replication, indicating that Zta is essential for virus viability (24). Zta has additional activities that are thought to indirectly facilitate viral DNA replication. These include the ability to block cell cycle progression (12, 13) and the disruption of the PML-associated nuclear domain 10 (ND10/PODs) (2, 8).

Modulation of Zta function can also play an important regulatory role in lytic cycle gene expression and DNA replication. Several cellular factors, including C/EBP (73, 74), p53 (83), NF-κB (34), c-Myc (38), CBP (2, 82), and uibinuclein (4), interact with Zta and cross-regulate each other’s activities. Most of these interactions have been mapped to the bZIP domain of Zta. Several amino acid residues in the bZIP domain have been implicated in distinct functions. Zta S186 has

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been shown to be essential for specific recognition and transcription activation of the Rta promoter (3, 27, 28). Mutations of S186 were also found to compromise Zta-meditated cell cycle arrest (59). The bZIP domain of Zta is capable of recognizing methylated DNA sequences with higher affinity than unmethylated DNA (10), and S186 contributes to this recognition specificity (9). Zta also possesses a notable cysteine residue, C189, that aligns with the highly conserved cysteine or serine residue found in most members of the bZIP superfamily (6). The orthologous cysteines in c-Jun and c-Fos are essential for redox regulation of DNA binding (1, 6, 75, 77). These cysteines confer sensitivity to oxidizing conditions which can result in disulfide bond formation in Jun and Fos (1, 6). The DNA binding activity of Zta is also sensitive to oxidation, and C189 has been presumed, but never formally shown, to be responsible for this sensitivity (6). Additionally, cysteine residues can be modified by nitrosylation through a signaling pathway involving the inducible cellular enzyme nitrous oxide synthase (iNOS) (26, 51). Nitrosylating agents and iNOS have been implicated in the inhibition of EBV lytic replication (30, 50), but the molecular targets of nitrosylation have not been identified. Here, we show that Zta C189 is required for lytic cycle replication through a mechanism that appears independent of DNA binding, transcription activation, or Rta interaction. We also demonstrate that C189 in combination with other cysteine residues confers DNA binding sensitivity to oxidation and nitrosylation.

**MATERIALS AND METHODS**

**Plasmids.** A C189S point mutation was generated by PCR mutagenesis with the QuickChange site-directed mutagenesis kit (Stratagene). Wild-type (wt) and C189S mutant cDNAs were cloned into the BamHI site of pQE8 bacterial expression vector (Qiagen) and confirmed by DNA sequencing. Additional serine substitution mutations at C222, C132, and C171 were introduced by serial modification using the QuickChange system and confirmed by DNA sequencing (Stratagene). Zta wt and C189S mutation EcoRI-Sall fragments were cloned in pX3FLAG-myc-CMV24 vector (Sigma) for mammalian cell expression. Rta expression vector pRTS15 was a gift of D. Hayward. Luciferase plasmids for Zta, C189S mutant cDNAs were cloned into the BamHI site of pQE8 bacterial expression vector pRTS15 was a gift of D. Hayward. Luciferase plasmids for Zta, C189S mutant cDNAs were cloned into the BamHI site of pQE8 bacterial expression vector (Qiagen) and confirmed by DNA sequencing. Additionally, cysteine residues can be modified by nitrosylation through a signaling pathway involving the inducible cellular enzyme nitrous oxide synthase (iNOS) (26, 51). Nitrosylating agents and iNOS have been implicated in the inhibition of EBV lytic replication (30, 50), but the molecular targets of nitrosylation have not been identified. Here, we show that Zta C189 is required for lytic cycle replication through a mechanism that appears independent of DNA binding, transcription activation, or Rta interaction. We also demonstrate that C189 in combination with other cysteine residues confers DNA binding sensitivity to oxidation and nitrosylation.

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**Transfections and reporter assays.** 293 cells were transfected using Lipofectamine 2000 (LF2000) reagent (Invitrogen) according to the manufacturer's protocol. Luciferase assays were performed by using the luciferase assay system (Promega). The results of luciferase assays were based on experiments performed in triplicate transfections. Expression levels of Zta were monitored by Western blot analysis.

**Measure of infectious virus.** Virus production was induced by transfection with mammalian cell expression plasmids of wild-type Zta and C189S into Zta knockout (ZKO) cells in 6-well plates. Supernatants were harvested from these cells 48 h posttransfection and passed into 6-well plates through a 0.8-μm filter. About 2 × 10^6 Raji cells in 50 ml of complete RPMI medium were added to each well containing the supernatants from different transfections. One-hundred ng/ml tetradecanoyl phorbol acetate (TPA) was also added to each well to stimulate green fluorescent protein (GFP) expression from recombinant Zta knockin virus. The cells were collected 4 days after infection by centrifugation and washed once with cold 1 × phosphate-buffered saline (PBS). About 5 × 10^6 cells were then resuspended in 0.5 ml of PBS for fluorescence-activated cell sorter (FACS) analysis. The virus titers were determined by analyzing the percentage of green fluorescent cells by FACS.

**Southern blot analysis of viral replication.** ZKO-293 cells were transfected with vector, Zta-wt, or ZtaC189S plasmid. After 6 h posttransfection, cells were resuspended in 45 μl of PBS and mixed with 45 μl of 2% low-melting-point agarose (Bio-Rad), pipetted into plug molds (Bio-Rad), and chilled. The agarose plugs were incubated for 24 h at 30°C in lysis buffer (0.2 M EDTA [pH 8.0], 1% sodium sarcosyl, 1 mg/ml proteinase K). The agarose plugs were washed twice in TE buffer (10 mM Tris [pH 7.5] and 1 mM EDTA). Pulsed-field electrophoresis was performed as described previously for 24 h at 14°C with a linear ramping pulse of 0.1 to 100 s through 120°C (Bio-Rad CHEF Mapper) (36). DNA was transferred to nylon membranes using established methods for Southern blotting (60). The DNA was then detected by hybridization with a 32P-labeled probe specific for the EBV oriLyt region and visualized with a Molecular Dynamics PhosphorImager.

**Chromatin immunoprecipitation (ChIP) assay and quantitative real-time PCR.** ChIP assays were performed essentially as described by Upstate Biotechnology with minor modifications (15). Cells were cross-linked with 1% formaldehyde and lysed in a buffer containing 1% sodium dodecyl sulfate (SDS), 10 mM EDTA, 50 mM Tris-Cl (pH 8.0). Chromatin was sonicated to ~600 bp and diluted with immunoprecipitation dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 20 mM Tris-Cl, pH 8.0, 167 mM NaCl) to a concentration of 500 ng/ml (or the equivalent of 1 × 10^7 cells/ml). Antibodies were added to 1 × 10^7 cells equivalents (1 ml of lysate). Antibodies used included rabbit polyclonal immunoglobulin G (IgG) (Santa Cruz Biotechnology), rabbit polyclonal Zta, mouse monoclonal Rta (Argene, Inc.), and FLAG-M2 (Sigma). Input (total) DNA was obtained from samples not incubated with antibody. ChIP DNA was purified proteins were quantitated by immunoblotting and Bradford assays and were used at equal concentrations for DNA binding assays.

**DNA binding assays.** Oligonucleotides probes containing Zta response elements (ZREs) were derived from EBV promoter sequences identical to those described in Bhende et al. (10). Rp-ZRE1 (forward, GATCTGCAATGGCTCATAAAAGA), Rp-ZRE2 (forward, GATCTGGAGTATGAGCGATT), and mutated Rp-ZRE2 (forward, GATCAAGCATTAGMCGATTAT), methylated Rp-ZRE2 (forward, GATCAAGGTTATAGCGMAGTTAT), and mutated Rp-ZRE2 (forward, GATCAAGCATTAGMCGATTAT), and methylated Rp-ZRE2 (forward, GATCAAGGTTATAGCGMAGTTAT), and mutated Rp-ZRE2 (forward, GATCAAGCATTAGMCGATTAT), and methylated Rp-ZRE2 (forward, GATCAAGGTTATAGCGMAGTTAT), and mutated Rp-ZRE2 (forward, GATCAAGCATTAGMCGATTAT). The annealed oligonucleotides were labeled with 30 μCi of [32P]dATP and 0.1 mM dCTP, dGTP, and dTTP using 2 U of Klenow enzyme (Roche) for 20 min at 37°C. Unincorporated nucleotides were removed on a Microspin G25 column (Amersham Biosciences). Protein extracts were diluted in 20 μl of DNA binding buffer (5 mM MgCl₂, 5 mM β-mercaptoethanol, 40 μg/ml poly(dI-dC)· poly(dI-dC), 500 μg/ml of bovine serum albumin, and 10^6 counts per million of radiolabeled duplex oligonucleotide). The DNA binding mixtures were incubated for 20 min at 37°C and electrophoresed in a 6% acrylamide gel at 110 V and visualized by a PhosphorImager.
analyzed by real-time PCR using an AB 7000 (Applied Biosystems). ChIP DNA and total viral DNA were quantitated using the standard curve method and calculations using AB Prism software (Applied Biosystems). All values were normalized to the respective input controls corresponding to each sample. These values were based on a standard curve, which includes serial dilutions of input DNA and a nontemplate control. The slope of this curve falls between 3.3 and 3.9, with points that fall within the linear range of DNA amplification but above the background threshold. Real-time primers were designed using Primer Express (Applied Biosystems) and include Rp (forward, CGGAAACCCTGCGAGACTAC; reverse, GCCCTGTCGTCGGGAGATA), OriLyt Hl (forward, TC GCCTTCTTTATCTTCTTTTG; reverse, CCCAGCAGGGCTAAAATGA CA), OriLyt Hr (forward, CGCGTGCCTTACTGACTTGTC; reverse, CCAGG AAGTGCGGAGCAT), and actin (forward, AACCACCCACACCACAAAG; reverse, CACTGACTTGAGACCAGACTGTTGAATAAAAA).

Reverse transcription-PCR (RT-PCR) analysis. RNA was isolated from 5 × 10⁶ cells using the RNeasy protocol (QIAGEN) and further digested with RNase-free DNase (Ambion) following the manufacturer’s instructions. RNA was eluted twice with 30 l RNase-free water each. A second step of DNase treatment was carried out prior to reverse transcription. Reverse transcription and conventional PCR were carried out using total RNA extracted from transfected ZKO cells with the following primers: BcLF1 (forward, TATGCCCAATCCCAAGTACACG; reverse, TGGACGGGTGGAGGAAGTCTTC), BLLF1 FIG. 1. A conserved bZIP cysteine (C189) is required for EBV lytic cycle replication. (A) Schematic of Zta functional domains and alignment of the bZIP domain of c-Jun, CREB, ATF1, c-Fos, GCN4, MafG, NF-E2-p45, C/EBP, and Zta. (B) Western blot of ZKO-293 cells transfected with Zta wt or C189S and probed with anti-Zta-specific antisera. (C) Virus production was measured 48 h after ZKO-293 cells were transfected with Zta wt, C189S, or vector control. Virus production was quantified by FACS analysis of GFP-positive Raji cells infected with supernatants from transfected ZKO cells. (D) DNA amplification was monitored by quantitative real-time PCR in ZKO-293 cells 24 h posttransfection with Zta wt (black) or C189S (gray). DNA amplification was monitored with primers specific for GAPDH, OriLyt regions (Hl or Hr), or at BRLF1 promoter (Rp). (E) Southern blot analysis of EBV DNA in ZKO-293 cells transfected for 36 h with vector, Zta-wt, or Zta-C189S. EBV DNA was isolated in agarose plugs and fractionated by pulsed-field electrophoresis. Linear (lower panel-short exposure) and episomal (upper panel-long exposure) EBV genomes are indicated.
A conserved cysteine in the Zta bZIP region is required for lytic cycle replication. Alignment of the Zta bZIP domain with other members of the bZIP superfamily revealed a strong conservation of either cysteine or serine in the residues aligned with Zta C189 (Fig. 1A). Zta C189 was mutated to serine and analyzed for its effect on viral lytic cycle DNA replication (Fig. 1). Zta wt and C189S were expressed at similar levels in ZKO-293 cells containing the EBV bacmid lacking the BZLF1/Zta open reading frame (24) (Fig. 1B). We assayed EBV lytic replication by three independent methods. First, we measured the production of infectious progeny virus after transfection of wt or C189S Zta. Production of GFP-positive virus was measured by superinfection of Raji cells followed by FACS quantification. We found that viral production was reduced 8-fold in C189S relative to wt Zta (Fig. 1C). Viral DNA replication was also measured by quantitative real-time PCR analysis of viral DNA relative to cellular DNA from vector control-transfected cells (Fig. 1D). We measured the average amplification of viral DNA at 24 h posttransfection at several regions of the viral genome, including OriLyt and Rp. Zta wt amplified viral DNA 20- to 40-fold in 24 h. In contrast, C189S had no more than a twofold amplification of viral DNA, indicating that this mutation blocked that ability of Zta to stimulate EBV lytic replication. Lytic replication was also monitored by Southern blotting of ZKO-293 cell DNA 36 h after transfection with vector, Zta wt, or Zta C189S (Fig. 1E). DNA was isolated by proteinase K-sarkosyl lysis of transfected cells embedded in agarose plugs and analyzed by pulsed-field electrophoresis to separate linear and circular forms of viral DNA. We found that Zta wt-transfected cells had a significant (10-fold) increase in linear DNA relative to cellular DNA from vector control-transfected cells (Fig. 1D). We measured the average amplification of viral DNA at 24 h posttransfection at several regions of the viral genome, including OriLyt and Rp. Zta wt amplified viral DNA 20- to 40-fold in 24 h. In contrast, C189S had no more than a twofold amplification of viral DNA, indicating that this mutation blocked that ability of Zta to stimulate EBV lytic replication. Lytic replication was also monitored by Southern blotting of ZKO-293 cell DNA 36 h after transfection with vector, Zta wt, or Zta C189S (Fig. 1E). DNA was isolated by proteinase K-sarkosyl lysis of transfected cells embedded in agarose plugs and analyzed by pulsed-field electrophoresis to separate linear and circular forms of viral DNA. We found that Zta wt-transfected cells had a significant (10-fold) increase in linear DNA relative to vector control, while Zta C189S-transfected cells had a much reduced amplification of linear DNA (2.5-fold).
Transcription activation by C189S. To investigate the mechanistic basis for the C189S defect in viral replication, we first analyzed protein levels of Zta and Zta-responsive viral genes in ZKO-293 cells at 24 h posttransfection with the wt or C189S mutant (Fig. 2A). Zta C189S induced expression of Rta and EA-D at a slightly reduced level relative to that of Zta wt. We next measured mRNA levels of several viral genes of different function and temporal expression patterns (Fig. 2B and C). For comparison, we compared the Zta C189S mutation with the S186A mutation, which has a known defect in transcription activation of BRLF1. We found that C189S activated BRLF1 and BHRF1 in a manner similar to that of Zta wt. C189S was slightly defective in activation of BBLF4, BSLF1, and BARF1 and was more significantly impaired in activation of BLLF1 and BcLF1. In contrast, S186A had no detectable transcription activation of any of these viral genes.

Since expression of some herpesvirus genes are partially dependent on DNA replication, it is possible that the C189S transcription defect can be attributed, in part, to a failure to stimulate DNA replication. Therefore, we examined the transcription activation properties of C189S on viral promoters removed from the context of the viral chromosome (Fig. 2D). Zta wt and C189S were compared for their ability to activate transcription of viral promoters fused to luciferase reporter constructs in EBV-negative 293 cell lines. We found that Zta C189S activated transcription of BRLF1, BZLF1, BHLF1, and BMRF1 as well as or better than Zta wt. Expression levels of Zta wt and C189S were nearly equivalent (Fig. 2A and data not shown).

The requirement for lytic DNA replication prior to late gene activation was further investigated using acyclovir to inhibit viral DNA polymerase and lytic replication. For these experiments, we analyzed viral replication and gene expression at 48 h posttransfection, when C189S stimulated low but detectable DNA replication (Fig. 3A). Addition of 500 μM acyclovir inhibited all detectable viral replication in ZKO-293 cells transfected with Zta wt or C189S as determined by real-time PCR analysis of viral DNA (Fig. 3A). Gene expression was first analyzed by Western blotting with antibodies to early gene products EA-D (BMRF1), Rta (BRLF1), and Zta (Fig. 3B). We found that Zta wt and C189S stimulated Rta and EA-D protein levels to nearly equal levels in untreated and acyclovir-
treated cells. We next compared the effect of acyclovir on mRNA levels of several viral genes using RT-PCR analysis (Fig. 3C). At 48 h posttransfection, we observed no significant difference between Zta wt and C189S in transcription activation for any of the tested viral genes. Interestingly, we found that acyclovir treatment strongly inhibited mRNA expression of BcLF4, BLLF1, and BARF1, weakly inhibited BSLF1 and BBLF4, and had no significant effect on BHRF1, BMRF1, and BRLF1. Transcription activation by Zta wt and C189S were almost indistinguishable in untreated and acyclovir-treated cells under these conditions. These results indicate that BcLF4, BLLF1, and BARF1 are true late genes that require DNA replication prior to transcription activation. These results also demonstrate that at later times posttransfection, C189S weakly stimulates replication and shows no significant defect in transcription activation of early or late genes.

**DNA binding properties of Zta C189S.** The DNA binding properties of highly purified Zta wt and C189S were compared in electrophoretic mobility shift assays (EMSA) with several ZREs. Zta wt and C189S were expressed in *E. coli* and purified to near homogeneity (Fig. 4A). Comparing identical amounts of Zta wt and C189S revealed that these two proteins bind AP1, Rp-ZRE1, and Rp-ZRE3 sites with nearly equal affinities. C189S did bind to Rp-ZRE2 with slightly lower affinity, suggesting that C189 contributes to some DNA recognition and stability. Similar results were observed when Zta wt and C189S proteins were isolated as native proteins from transfected mammalian cells rather than from *E. coli* (data not shown). Thus, Zta C189S can bind DNA in vitro with similar affinity to Zta wt but may have subtle differences in affinity for some specific sites, like ZRE2.

Recent reports revealed that Zta can bind to methylated ZRE sites with higher affinity than to unmethylated ZREs (9, 10). We next compared the ability of Zta wt and C189S to bind to methylated ZRE2 using EMSA. In our initial studies, we did not find significant differences in the affinity of Zta proteins for methylated or unmethylated ZRE2-radiolabeled probes (Fig. 5A and B). However, when radiolabeled ZRE2 was challenged with cold competitor DNA, we found that Zta had higher affinity for methylated ZRE2 than for unmethylated ZRE2 (Fig. 5C, compare lane 8 to 10 and 3 to 5). A similar increased affinity for methylated ZRE2 was observed for C189S (Fig. 5D). This difference in direct binding relative to competitor challenge may reflect differences in the kinetic properties of DNA binding by Zta. Nevertheless, we conclude that the C189S replication defect is not caused by a failure to recognize methylated ZREs.

It is possible that DNA binding in vitro may not reflect the DNA binding properties observed in vivo. The ChIP assay was used to determine if Zta C189S was defective for DNA binding in vivo. ZKO-293 cells were transfected with cytomegalovirus (CMV)-FLAG-Zta wt, CMV-FLAG-Zta C189S, or control CMV-FLAG vector and assayed by ChIP with FLAG antibody or control IgG (Fig. 6). We assayed binding at the BRLF1 promoter Rp (Fig. 6A) or at OriLyt (Fig. 6B). We observed significant binding of FLAG-Zta wt and FLAG-Zta C189S to both Rp and OriLyt. The percentages of input DNA bound by Zta wt and C189S were similar, indicating that these two proteins have similar binding activities in vivo.

**Zta C189S cannot be rescued by Rta.** The S186A mutation of Zta has been shown to be defective in transcription activation of Rta, and overexpression of Rta can rescue lytic activation of S186A (3). To determine if Zta C189S had a defect similar to that of S186A, we tested the ability of Rta to rescue the replication defect of Zta C189S (Fig. 7A). Quantitative real-time PCR analysis of EBV DNA was measured for ZKO-293 cells transfected with Zta wt or C189S in the absence or presence of Rta. We found that Rta cotransfection did not significantly increase viral DNA replication with Zta wt or C189S. This indicates that the Zta C189S replication defect cannot be rescued by overexpression of Rta and is therefore mechanistically distinct from the S186A defect in Rp transcription activation.

**Zta C189S is weakly impaired for coimmunoprecipitation with Rta.** Zta has been reported to interact with Rta in vivo, and it is possible that the failure of Zta C189S to activate replication is a consequence of a failure to interact with Rta. The interaction of Zta with Rta was measured by coimmuno-
precipitation assay of Flag-tagged Zta wt and C189S in ZKO-293 cells (Fig. 7B). Relative to vector control-transfected cells, Zta C189S stimulated expression of Rta similar to that of Zta C189S. Interestingly, we found a slight reduction in Rta coimmunoprecipitating with C189S relative to that precipitated with Zta wt. No Rta was detected in immunoprecipitations with control IgG antibody. Similar levels of Zta wt and Zta C189S were precipitated using the FLAG antibody (lower panel). Thus, C189S is weakly impaired in its ability to interact with Rta in transfected ZKO-293 cells.

**Rta binds OriLyt in C189S-expressing cells.** Zta may influence Rta binding to several sites in the EBV genome. To determine if C189S may be defective in this property, we used the ChIP assay to monitor the in vivo DNA binding properties of Zta and Rta at EBV OriLyt (Fig. 7C and D). We found that Rta bound to OriLyt indistinguishably in ZKO-293 cells transfected with Zta wt or C189S (Fig. 7C). Ectopic expression of Rta increased the amount of Rta binding in both wt and C189S-transfected cells, with slightly more binding in Zta wt. ChIP analysis of Zta protein at OriLyt indicated that C189S bound as well as or better than Zta wt (Fig. 7C). Ectopic expression of Rta did not have a significant effect on Zta wt or C189S binding in these assays (Fig. 7D). These results indicate that Zta and Rta binding to OriLyt are nearly identical in wt or C189S-transfected ZKO cells.

**C189 and other cysteine residues are sensitive to redox regulation of DNA binding.** The DNA binding activity of Zta, like c-Jun, requires reducing agents, like DTT and β-mercaptoethanol, to protect against cysteine oxidation and disulfide bond formation. Based on studies with c-Jun, we predicted that the Zta C189S would be resistant to oxidation. We found that C189S provided a small but measurable protection from cysteine oxidation (Fig. 8A). In the absence of any reducing agent, we found that Zta wt was completely incapable of binding DNA (Fig. 8A, lane 1). In contrast, Zta C189S had a detectable but unstable binding in the absence of DTT (Fig. 8A, lane 10). C189S also demonstrated a slight but potentially significant resistance to the cysteine nitrosylating reagent SNAP (compare lane 3 to 9 and 12 to 18). To determine if other cysteine residues in Zta conferred resistance to oxidation, we combined C189S and C222S, a second serine residue in the dimerization region of Zta. C189/222S bound DNA, albeit with unstable smearing, in the absence of reducing agent (Fig. 8B, lane 10). C189/222S also showed increased resistance to SNAP relative to the wt or C189S alone (lanes 12 to 18). Two additional cysteine residues amino terminal to the Zta bZIP domain were mutated to serines, creating the quadruple cysteine substitution mutant C189/222/132/171S. This mutant of Zta was found to bind DNA efficiently in the absence of DTT (Fig. 8C, lane 10) and to be highly resistant to SNAP (lanes 12 to 18). Inter-
sensitive DNA binding of Zta. 

several cysteine residues contribute to the redox- and SNAP- oligomerization state of Zta. These findings also indicate that C189S, suggesting that these cysteine substitutions altered the quadruple cysteine mutant had slower mobility than Zta wt or 
estingly, the Zta-DNA complex formed by C189/222S and the curve method and is presented as the percentage of input DNA.

FIG. 6. C189S binds OriLyt and Rp in vivo. ZKO-293 cells trans- 
fected with CMV-FLAG vector (white), CMV-FLAG-Zta wt (black), 
or CMV-FLAG-Zta C189S (gray) were assayed by ChIP with anti- 
FLAG antibody and quantitative real-time PCR with primers specific 
for Rp (A) or OriLyt (B). ChIP DNA was quantitated by the standard 
curve method and is presented as the percentage of input DNA.

DISCUSSION

In this work, we show that the conserved cysteine residue C189 in the bZIP domain of Zta was required for EBV lytic cycle replication (Fig. 1). Substitution mutation of C189 to serine (C189S) severely impaired Zta-mediated lytic replication as measured by infectious virus production (Fig. 1C), quantitative real-time PCR analysis (Fig. 1D), and Southern blotting of viral genomes (Fig. 1E). The mechanistic basis for this defect was investigated by several different approaches. We found that C189S was capable of activating viral early gene expression but was impaired for activation of late viral mRNA relative to wt Zta (Fig. 2). This failure to activate late genes was consistent with a failure to initiate lytic cycle DNA replication, and we show that genes not activated by C189S were inhibited when DNA replication is blocked by acyclovir treat-
with the KSHV bZIP protein K8 (63). K8 is essential for KSHV lytic replication, but its mechanism of action is also unknown (47, 81). Both Zta and K8 share the ability to interact with C/EBP, and C/EBP binding sites can be found throughout the KSHV OriLyt (47, 71, 74). We were not able to detect a strong interaction between Zta and C/EBP in ZKO-293 cells (data not shown) and therefore could not assess the effect of C189S on the C/EBP interaction. Interestingly, C/EBP possesses a serine at the C189 position, suggesting that it is not subject to the same cysteine-dependent regulation as Zta (Fig. 1A). Thus, while Zta and K8 most likely provide similar essential functions in viral lytic replication, the mechanisms governing the cysteine-dependent regulation through C189 have apparently not been conserved between these two virus families.

**DNA binding properties of Zta.** The bZIP domain of Zta is most similar to C/EBP, a protein named for its degenerate sequence recognition capabilities. The DNA binding properties of Zta have been explored in detail previously (63). Like C/EBP, Zta recognizes a wide and degenerate array of sequence elements (45, 46). In addition to binding diverse primary sequences, Zta and C/EBP have high affinity for oligonucleosomal DNA (16). In addition to binding oligonucleosomes, Zta can also preferentially bind to some methylated DNA sequences (10). The S186A mutation has been shown to have a pronounced defect in transcription activation of the endogenous BRLF1 (Rta) promoter. The primary defect in S186A can be attributed to its failure to recognize methylated ZREs in the Rta promoter (3, 9, 10). We did not observe any significant failure of C189S to bind to methylated ZREs in vitro or to bind to viral DNA in vivo using ChIP assays (Fig. 7D), nor did we find that Rta coexpression could rescue the Zta C189S defect in DNA replication (Fig. 7A). These findings indicate that the defect in C189S is mechanistically distinct from the defect in S186A. We also did not observe any defects in C189S in the ability to bind oligonucleosomal DNA (data not shown). Thus, DNA recognition of methylated DNA or chromatin structure does not appear to account for the defect of Zta C189S in stimulating lytic cycle DNA replication.

**Potential regulation of EBV replication by C189 modifications.** Posttranslational modifications of Zta may play an important role in regulation of lytic cycle gene expression and DNA replication. Zta can be sumoylated on lysine 12, and mutation of lysine 12 to alanine causes a severe defect in lytic cycle DNA replication without any other obvious defects in transcription activation (2, 20). Zta can be phosphorylated by casein kinase II, which can influence its ability to activate late gene transcription (22). TPA-inducible phosphorylation of S186 may be important for recognition of DNA binding sites in the Rta promoter (7), although phosphorylation of this residue remains controversial (21). It seems plausible that C189 may be subject to posttranslational modifications that regulate Zta DNA binding and lytic replication. Our data indicate that oxidation and S-nitrosylation inhibit Zta DNA binding in vitro (Fig. 8). Nitrosylation of cysteines is known to regulate the DNA binding properties of several cellular transcription factors, including NF-κB (54, 55), p53 (53), and replication protein A (RPA) (70). Modification of Zta C189 by oxidation or nitrosylation may inhibit DNA binding, which is likely to in-
Our data demonstrate that C189, along with several other cysteines, provides a mechanism for the negative regulation of Zta DNA binding. However, it is not clear how this negative regulation plays a role in the positive regulation of lytic replication. A similar mutation in the redox-sensitive c-Fos cysteine C154S enhances DNA binding and causes a corresponding increase in growth-transforming activity (57). Several possible explanations may account for the paradoxical redox sensitivity of Zta C189 and its requirement for viral DNA replication. S-nitroso or related cysteine-dependent posttranslational modification of C189 may be required for protein complex assembly at OriLyt. A similar posttranslational regulation of Zta at K12 may be involved in facilitating lytic DNA replication. Alternatively, inhibition of Zta binding by oxidation or nitrosylation of C189 may be an essential step in replication initiation and fork progression at OriLyt. Finally, it is possible that the redox-sensitive cysteine in Zta is regulated by the REF1/APEX protein (29, 76, 78, 80). REF1/APEX is a bifunctional protein that protects c-Fos and c-Jun from oxidation and performs essential functions as an apurinic endonuclease in cellular base excision repair (77, 79, 80). REF1/APEX may interact or modify Zta and contribute to changes in OriLyt necessary for lytic cycle DNA replication. However, our data clearly indicate that other cysteine residues in Zta are involved in the redox-sensitive DNA binding activity (Fig. 8), and C189 may play a more important role in DNA recognition or binding to an essential factor required for lytic cycle replication. While the precise mechanism of C189 function in DNA replication remains unknown, our findings raise the possibility that Zta performs more complex functions in lytic cycle replication than previously appreciated.

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