Mutation of a Single Amino Acid Residue in the Basic Region of the Epstein-Barr Virus (EBV) Lytic Cycle Switch Protein Zta (BZLF1) Prevents Reactivation of EBV from Latency

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Zta, the product of the BZLF1 gene carried by Epstein-Barr virus (EBV), is crucial for reactivation of EBV from latency. Zta is a member of the bZIP family of transcription factors, and in common with many of these, Zta possesses a conserved cysteine residue in its basic region (C189) and a further cysteine residue in its ZIP region (C222). We demonstrate that C189 is required to reactivate EBV from latency but C222 is not and that this single amino acid affects two independent functions of Zta, (i) binding to a Zta-responsive site and (ii) manipulating the cell cycle.

Epstein-Barr virus (EBV) is associated with several human tumors, including Burkitt’s lymphoma, Hodgkin’s disease, and nasopharyngeal carcinoma (as reviewed in reference 6). Once a person is infected with EBV, the virus persists for life due to its ability to establish latent infection in B lymphocytes. The switch into the lytic cycle, responsible for the production of new virions, is controlled by the viral gene BZLF1, encoding the protein Zta (7, 20, 26–28, 30). Zta is considered to belong to the leucine zipper (bZIP) family of transcription factors despite lacking the heptad repeat of leucines that is characteristic of the dimerization region of these proteins. In common with canonical members of the bZIP family, Zta contains a transactivation domain, a basic (DNA-binding) region, and a coiled coil (ZIP) region. In common with many bZIP members, such as c-fos and c-jun (1, 2), Zta contains a highly conserved cysteine located within the basic region (C189). Zta also possesses a further cysteine in the bZIP region within the coiled coil (C222). Interestingly, cysteine 189 and cysteine 222 are 100% conserved in all EBV strains that we have investigated (Akata, Jijoye, Mutu, and Rael and from nine patients with infectious mononucleosis [14 and unpublished data]). The redox state of the bZIP domain dictates the DNA-binding function of a subset of bZIP proteins: GCN4, c-fos, and c-jun (2). In contrast to these bZIP proteins, the DNA-binding function of C/EBPα is insensitive to the redox environment (2). It is of particular significance here that C/EBPα contains a serine residue in place of the usual cysteine residue, suggesting that the replacement of the cysteine in the basic region with a serine renders GCN4, c-fos, and c-jun insensitive to redox change. Experiments have shown this to be so (1, 2). Since the DNA-binding function of Zta is also redox dependent (2), we investigated the functional role of the two cysteines in the bZIP domain of Zta (C189 and C222) for the most critical function of Zta: the ability to reactivate EBV from latency.

Latency reactivation is compromised by a mutation of C189S in the basic region of Zta. Site-directed mutagenesis was used to introduce serine residues in place of the two cysteine residues, 189 and 222, within the context of the full-length Zta coding sequence. The resulting proteins (Zta C189S and Zta C222S) were expressed at equivalent levels in both in vitro expression systems and human cells (Fig. 1 and 2), and both localized appropriately to the nucleus (data not shown).

The ability of each mutant to induce the EBV lytic replication cycle was assessed following expression in 293-BZLF1-KO cells (7). In this system, using quantitative PCR to detect the viral genome, replicated viral DNA can be clearly observed 96 h after transfection of a wild-type Zta-expressing construct (Fig. 1). Expression of Zta C189S resulted in no increase in the basal level of viral DNA compared to the amount found in control cells, whereas expression of Zta C222S was able to induce replication of viral DNA to a level equivalent to that seen following the expression of wild-type Zta.

From this analysis it is apparent that C189 plays a critical role in the reactivation of the viral lytic replicative cycle, whereas C222 is dispensable for this function of the protein. We therefore sought to investigate the molecular basis for the dependence on C189 for the activation of EBV from latency by examining the effect of this mutation on two fundamental functions of Zta, the ability to form dimers and the ability to bind to DNA.

Neither C189 nor C222 influences the ability of Zta to form stable dimers. Zta folds into a dimer through a coiled coil motif (14), which is considered to be necessary to orient the two basic regions appropriately for interaction with DNA. The ability of a synthetic peptide spanning the coiled coil region of Zta to fold as a coiled coil has been measured previously using...
the biophysical technique of circular dichroism spectroscopy (CD) (14). Here, we generated an equivalent synthetic peptide containing the C222S mutation and undertook a comparable experiment. This revealed that the C222S peptide folds as a multimeric coiled coil, with a stability similar to that of wild-type Zta peptides (Fig. 2 and data not shown). The peptides did not include the basic region of Zta, so the impact of C189 on coiled coil stability could not be investigated in this manner. We therefore undertook further experiments to investigate the impact of the mutations of C189 and C222 on the ability of Zta to fold as stable dimers in the context of the full-length protein (Fig. 2). In vitro-translated Zta proteins were cross-linked by exposure to glutaraldehyde (as described in reference 33), and their state of association was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Our results showed that both Zta C189S and Zta C222S readily formed dimers at a level equivalent to that of the wild-type protein. As expected, H199ter, a deletion mutant of Zta that lacks the entire ZIP region, fails to form dimers in this assay. Thus, neither mutation appears to compromise the ability of Zta to dimerize.

**Mutation of C189 does not influence the ability of Zta to interact with three ZRE sites.** Initial experiments investigating the impact of C189 and C222 on the ability of Zta to bind to DNA revealed that both appeared as competent as the wild type (data not shown). Since C189 lies in the basic region of Zta, a series of electrophoretic mobility shift assay (EMSA) experiments were undertaken to question whether there are any subtle changes in the ability of Zta C189S to interact with DNA. Three different ZRE sites were assessed: AP1, ZRE, and OriLyt. An EMSA was also performed using a mutant version of the AP1 site in order to demonstrate DNA-binding specificity. We found that Zta C189S was able to bind to all ZREs tested as efficiently as wild-type Zta (Fig. 2). In fact, compared to the relative binding of wild-type Zta, Zta C189S bound 117.5% to the ZRE site, 99.5% to the AP1 site, and 140% to the OriLyt site. No DNA-protein complex was observed using the mutant AP1 probe. These results suggest that the inability of Zta C189S to activate the lytic cycle of EBV is not due to a failure of DNA binding, at least not to these three ZREs.

**C189 is critical for transactivation through ZREs in vivo.** From our results, we can conclude that Zta C189S is unable to induce the viral replicative cycle in vivo but can undertake the fundamental functions of Zta, dimerization and DNA binding, as efficiently as wild-type Zta in vitro. It is therefore likely that C189 is critical for a function of Zta that is only manifest in vivo. To address this, we investigated the ability of Zta C189S to transactivate gene expression through a ZRE-dependent reporter construct in a B-cell line. It is apparent from Fig. 3 that both wild-type Zta and Zta C222S are able to transactivate a reporter construct containing multiple copies of the ZREIIIB element (15- and 17-fold, respectively), whereas Zta C189S is able to achieve less than twofold activation. This appears to support our suggestion that C189 is required for a function that is only apparent in vivo and points toward a role for C189 in interaction with the Zta transcriptional activation mechanism. Since we had not previously examined Zta C189S binding to the particular ZRE (ZIIIB) used in the reporter construct, we examined whether Zta C189S recognition of this
site was impaired. Surprisingly, it appeared that Zta C189S was partially compromised in binding to the ZIIIB site (60%) in comparison to wild-type Zta (Fig. 3C to E). This preparation of Zta and Zta C189S bound to OriLyt and ZRE1 equally, in comparison to wild-type Zta (Fig. 3C to E). This preparation of Zta and Zta C189S bound to OriLyt and ZRE1 equally.

We then assessed the ability of Zta C189S to activate endogenous viral promoters in vivo in comparison to wild-type Zta. The control vector (pBABE), pBABE Zta, and pBABE Zta C189S were introduced into Raji cells. The expression of Zta target genes BMRF1 and BMRF1, after 24 h, was analyzed by quantitative PCR (Fig. 4B and C, respectively). The in vivo analysis of gene expression clearly showed a deficiency for Zta C189S in activating the expression of BMRF1 (Fig. 4B) and BMRF1 (Fig. 4C) in comparison to wild-type Zta. Additionally, Zta C189S was also deficient in binding to the “M” site located on the BMLF1 promoter (EB2) compared to the relative binding of wild-type Zta. Zta C189S binding to the “M” site was 75% of wild-type binding (data not shown). This correlates with its deficiency in activating BMLF1 gene expression in vivo. Consequently, the deficiency of Zta C189S to bind to a subset of ZREs may be linked with the reduction in transactivation function. Although it is a relatively small reduction in binding efficiency, this could be sufficient to explain the inability of Zta C189S to reactivate EBV from latency.

C189S is unable to mediate cell cycle arrest but can still interact with C/EBPα. Previous work suggested that the replication of EBV is associated with cell cycle arrest in many cell types (4, 5, 9) but that this is not a universal effect (18, 19). Consequently, it was shown that Zta could promote cell cycle arrest through multiple pathways (24, 25, 32), some of which required the basic region. We therefore determined the effects of C189 on cell cycle arrest. Expression vectors were introduced into HeLa cells (4, 5), and the effect of a range of expression of Zta was determined by fluorescence-assisted cell sorter analysis (Fig. 5). We observed an increase in cells in the G1 phase of the cell cycle but not in the G2 or M phases in these experiments. This revealed that a threshold level of Zta is required in order to mediate cell cycle arrest. However, once this has been exceeded, a similar percentage increase in G1 occurs at all levels of expression (Fig. 5B). The ability of Zta C189S to promote cell cycle arrest was then compared to that of wild-type Zta in HeLa cells. Strikingly, our results showed that Zta C189S had a much reduced ability to cause an increase in the G1 phase of the cell cycle (Fig. 5D).

Since previous studies have demonstrated that an interaction between Zta and C/EBPα occurs through the bZIP region and this interaction is required to promote cell cycle arrest (32, 33), we postulated that the inability of Zta C189S to promote cell cycle arrest could be due to a defect in its ability to interact with C/EBPα. The abilities of wild-type Zta and Zta C189S to interact with C/EBPα were therefore compared (Fig. 5E and F). The full-length coding sequence of C/EBPα was cloned into the pRSETA vector, which contains a polyhistidine tag. The resulting protein, His-C/EBPα, and the protein derived from the vector alone, His, were generated in Escherichia coli and purified using nickel beads. The coated beads were then mixed with proteins generated in an in vitro translation system. C/EBPα was used as a positive control (since C/EBPα forms homodimers), and luciferase was used as a negative control. As illustrated in Fig. 5E, wild-type Zta, Zta C189S, and C/EBPα bound to the His-C/EBPα beads, whereas luciferase did not bind significantly. The extremely low affinity of proteins for the His beads set the background of the assay. The results obtained are summarized by a histogram (Fig. 5F), showing no signifi-
cant difference between the interaction of wild-type Zta and Zta C189S with His-C/EBPα.

Here we provide further evidence that amino acids in the basic region of the EBV lytic cycle switch protein Zta are required to reactivate EBV from latency. Specifically, we show that C189 is essential for this function of Zta. Previous results have shown that substitution of the entire basic region of Zta for the residues encoded by the basic region of c-fos destroys this function of Zta (10) and that mutation of a neighboring serine residue (S186) to alanine also prevents reactivation of EBV from latency (10).

Our investigations into the mechanism by which C189 contributes to the disruption of latency revealed that two functions of Zta rely on C189. First, the ability to bind to a subset of ZREs was shown to be partially dependent on C189, resulting in decreased transactivation of a dependent promoter. Secondly, the ability to promote cell cycle arrest was shown to have an absolute requirement for C189. Interestingly, since cell cycle arrest can occur in the absence of DNA binding (dbm1 and dbm2 [25]), the two functions that we show to be dependent on C189 are independent of each other. This led us to question the possible mechanisms by which these defects would influence the reactivation of EBV from latency.

FIG. 3. Cysteine 189 is required for full transactivation activity of Zta. DG75 cells ($1\times10^6$) were transfected with 10 μg of a ZRE-dependent chloramphenicol acetyltransferase (CAT) construct (ZIIIBCAT) (17) together with 5 μg pRL-TK as a transfection control (Promega) and the pBabe series of expression vectors encoding Zta, the indicated mutants, or the “empty” expression vector. All experiments were undertaken in duplicate. (A) Forty-eight hours following transfection, soluble protein extracts were prepared and CAT and luciferase activities were determined. The relative expression of the ZIIIBCAT expression vector was determined by standardizing the signal from CAT to that from luciferase for each transfection (as described in reference 13). (B) Forty-eight hours following transfection, total protein extracts were prepared, fractionated on SDS-PAGE, and subjected to immunoblotting with a monoclonal antibody directed against Zta (BZ1) and one against Cdk2. EMSA was performed in order to assess the ability of Zta C189S and C/EBPα to bind to the ZREIIIB site in comparison with wild-type Zta. The ZIIIB oligonucleotides (GTACATTAGCAATGCCTG) were synthesized and processed as described in Hicks et al. (14). The proteins were translated, analyzed by SDS-PAGE (C), and quantitated, and then equal amounts were assessed by EMSA (D). Experiments were repeated in triplicate using different protein concentrations. The mean binding of Zta and Zta C189S to the ZIIIB site is summarized by a histogram along with the standard error (E). IVT, in vitro translation.
Fig. 4. Zta C189S is compromised in activating the expression of the Zta target genes BMRF1 and BMLF1 in vivo. Raji cells (5 × 10⁶) (23) were transfected with 3 μg of pBABE (Control), pBABE Zta, or pBABE C189S using Amaxa nucleofector T solution (G-16 program; Amxam Biosystems). Twenty-four hours later, total RNA was extracted using an RNeasy kit (Qiagen) followed by the preparation of cDNA using the ImProm-II Reverse Transcription System (Promega). Quantitative real-time PCR was performed using the cellular ribosomal gene, L32 (5'-CAAATTTGATGGATCTTA-3') and 5'-TGA CGTTGTCAGACCAAACT-3'), to assess the cDNA preparation for each sample. Zta primers (5'-CTATCAGGACCTGGAGGG C-3' and 5'-CACACGACACAGGAAGG-3') were used in order to compare the transfection efficiency between pBABE (control vector), pBABE Zta, and pBABE Zta C189S (A). Expression of both BRLF1 (B) and BMLF1 (C) was assessed for each transfection using quantitative real-time PCR followed by normalization for cDNA efficiency. This experiment was carried out in duplicate. A. Histogram summarizing the transfection efficiency (with standard errors) of pBABE and pBABE Zta C189S relative to pBABE Zta. B. Histogram illustrating the expression of the BMRF1 gene (5'-GGACCTGCCT GTGGATCTTA-3' and 5'-TCTTCGGAGAACCGTGGTTAAA-3') relative to pBABE Zta along with standard errors. C. Histogram representing the expression of the BMLF1 gene (5'-AGAATGGCCCTGA CAAGTCG-3' and 5'-CACACGATGAATCGGCGAATG-3') relative to pBABE Zta with standard errors.

The DNA-binding function of Zta probably contributes to its ability to initiate and complete the viral lytic cycle. Specifically, Zta interacts with ZREs in several immediate-early and early viral genes (27, 29) and with the origin of lytic replication (27, 29). Since the DNA-binding function of Zta is reduced to 60 to 75% of wild-type efficiency for a subset of ZREs in vitro, we predict that C189 also influences the DNA-binding efficiency of Zta in vivo. Elucidation of molecular mechanisms governing the observed reduction in DNA-binding function to a subset of ZREs must await detailed information on the structure of Zta bound to DNA. However, it is worth noting that the cysine equivalent to C189 in the solved structure of GCN4 is located in very close contact with DNA (22), so it is reasonable to propose that C189 might be compromised in DNA-binding function in a base-specific manner, thus explaining why only a subset of ZREs are affected. In cells, the recognition of different ZREs might be further influenced by the methylation state of the DNA, as Zta has recently been shown to interact with some sites far better if they contain a methylated CpG residue (3). In a cell infected with wild-type EBV, autoamplification of Zta expression through the ZIIB element in the Z promoter by Zta is considered to be a crucial step in the lytic cycle (8), and our data imply that C189 contributes to this step. However, this is not sufficient to explain the observed inability of Zta C189S to induce the viral lytic cycle in 293-BZLF1-KO cells, since the knockout mutation of Zta within this virus renders the autoamplification loop redundant (7).

We have shown that the binding of Zta to the “M” site, a ZRE within the promoter of the BMLF1 gene, which is expressed early in the viral lytic cycle, is also partially dependent on C189. From this, we predicted that expression of EB2, the product of the BMLF1 gene, will be dependent on C189. Experiments in Raji cells confirmed this prediction in vivo and furthermore showed that the expression of BMRF1 was similarly dependent on C189. As a viral knockout, BMRF1 has recently been shown to be compromised in producing infectious EBV (12). We propose that the reduced interaction of Zta C189 to ZREs will compromise at least one essential step in the viral lytic cycle.

The requirement of C189 for Zta to manipulate the cell cycle may also contribute to the requirement for C189 to achieve latency reactivation. Cell cycle arrest is observed in a number of, but not all, cell lines following expression of Zta (4, 5, 9, 19). Analysis of cell cycle distribution by staining for DNA content by fluorescence-assisted cell sorting initially suggested that this occurs primarily in the G1 phase of the cell cycle (4, 5, 9), but more recent investigations suggest that it can also occur within the S phase (18, 19) and that S-phase-specific enzymatic activities are present in the arrested cells (15, 16, 31). Thus, while an arrest in cell cycle progression is consistently observed during the viral lytic cycle, this occurs at a typical cell cycle checkpoint or whether it is a tailor-made checkpoint adapted by EBV is currently unclear. However, it is likely that EBV manipulates the cell cycle of cells undergoing the lytic cycle for two reasons, (i) to ensure the expression of cellular genes required for viral DNA replication and (ii) to prevent mitosis occurring in a cell before the completion of the viral lytic cycle.

The mechanism governing the defect in cell cycle manipulation by Zta C189S is less clear. It has been proposed that C/EBPα is required and that a physical interaction between Zta and C/EBPα plays a major role (32, 33). We explored the possibility that this interaction is dependent on C189 but showed this to be untrue, at least in vitro. However, data from Rodriguez et al. and Wu et al. show that cell cycle arrest can be promoted by a mutant of Zta, dbm1, that is unable to interact with C/EBPα (25, 32), so the universal dependence on C/EBPα to promote cell cycle arrest is in doubt.

The inability of Zta C189S to reactivate EBV from latency correlates with a reduction in two functions of Zta, and the question remains whether either or both are responsible for causing the defect in latency reactivation. This will only be resolved if mutations that affect both functions individually can be identified and their effect on reactivation of EBV from latency assessed. However, it is probable that both DNA binding to ZREs and cell cycle manipulation are required to reanimate EBV from latency.

The dependence of two such diverse functions on a single amino acid residue of Zta highlights that the basic region of Zta is not only required for interaction with DNA but might also act as an interaction face for other molecules in the DNA-bound or unbound state.
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FIG. 5. Ability of Zta to promote cell cycle arrest depends on cysteine 189. HeLa cells (between 1 × 10⁶ and 8 × 10⁶) were transfected with pBabe-Zta (between 1 µg to 30 µg), pBabe-Zta C189S (between 5 µg to 20 µg), or pBabe alone (between 5 µg to 30 µg) using calcium phosphate (4). Cells were harvested after 48 h or 96 h. (A) The optimal amount of Zta was assessed by titrating the amount of the pBabe-Zta expression vector and was analyzed by Western blotting with BZ1 monoclonal antibody and cdk2 as a loading control. (B) The change in the percentage of cells in the G₁ phase of the cell cycle was determined in relation to the empty vector alone (G₁ increase), which was assessed by propidium iodide staining and flow cytometry. (C) Western blot analysis showed the protein levels after HeLa cell transfection prior to flow cytometry analysis. (D) A histogram showing the mean percent increase in G₁ with the standard error from four independent experiments is shown. (E) To assess the ability of C189S to interact with C/EBPα, a His-tagged version of full-length human C/EBPα was generated, expressed in BL21 pLys S cells, and purified using nickel beads. Sixty microliters of the resulting beads, which corresponds to the His vector alone (R) or His-C/EBPα (C), was mixed with equal amounts of in vitro-translated proteins in phosphate-buffered saline supplemented with 600 mM NaCl and 20 mM imidazole. Following a 1-h incubation at 4°C, the beads were extensively washed in the same buffer and then fractionation on SDS-PAGE followed by phosphorimager analyses. (F) Histogram showing the means of two independent experiments with standard errors.
controlled by the co-operative functions of two transactivators. EMBO J. 19:3080–3089.


