The reversal of epigenetic silencing of the EBV genome is regulated by viral bZIP protein

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Abstract
EBV (Epstein–Barr virus) alternates between latency and lytic replication. During latency, the viral genome is largely silenced by host-driven methylation of CpG motifs and in the switch to the lytic cycle this epigenetic silencing is overturned. A key event is the activation of the viral protein Zta with three ZREs (Zta-response elements) from the BRLF1 promoter (referred to as Rp). Two of these ZREs contain CpG motifs and are methylated in the latent genome. Biochemical analyses and molecular modelling of Zta bound to methylated RpZRE3 indicate the precise contacts made between a serine and a cysteine residue of Zta with methyl cytosines. A single point mutant of Zta, C189S, is defective in binding to the methylated ZREs both in vitro and in vivo. This was used to probe the functional relevance of the interaction. ZtaC189S was not able to activate Rp in a B-cell line, demonstrating the relevance of the interaction with methylated ZREs. This demonstrates that Zta plays a role in overturning the epigenetic control of viral latency.

EBV (Epstein–Barr virus)
Infection with EBV is common throughout the human population and EBV is the causative agent of infectious mononucleosis and is linked to malignancies such as endemic BL (Burkitt’s lymphoma), NPC (nasopharyngeal carcinoma) and HD (Hodgkin’s disease) [1–4]. EBV infects and establishes long-term latency in B-lymphocytes [5]. In BL cells, the EBV genome is heavily methylated and few viral genes are expressed [6–14]. Methylation of DNA is generally associated with inhibition of gene expression. This is mediated in part by the association of specific methyl-CpG-binding proteins with methylated DNA, leading to transcriptional silencing and chromatin remodelling [15], and in part by the inhibition of DNA binding of some transcription factors through methylation of DNA [16].

Viral bZIP (basic leucine zipper) transcription factor
Zta is a multifunctional protein that resembles a bZIP transcription factor [17–24]. Zta is the first viral gene expressed during the switch from latency to lytic replication and it activates the expression of a second viral transcription factor, Rta, encoded by BRLF1 [25]. Both of these transcription factors are essential for viral replication and together promote the expression of the remaining viral lytic genes [26]. Zta interacts directly with sequence-specific DNA ZREs (Zta-response elements) [19]. The sequences of these resemble AP1 (activatory protein 1) and C/EBPα (CCAAT/enhancer-binding protein α) recognition elements, but Zta is unusual in recognizing a wide range of variants of the core ZRE sequence. The solution of the crystal structure of Zta interacting with a canonical AP1 site revealed that the interaction is stabilized by many contacts between Zta and the sugar phosphate backbone of DNA, rendering specific interactions with bases less critical than for some other transcription factors [21,27]. The BRLF1 promoter, referred to as Rp, contains three ZREs, two of which (RpZRE2 and RpZRE3) include CpG motifs that are subject to methylation [3]. A pivotal study by Shannon Kenney’s group demonstrated that the interaction of Zta with RpZRE2 and RpZRE3 is enhanced by methylation [28].

We propose that ZREs can be divided into three functional classes (Table 1). Class I ZREs do not contain a CpG motif and so the interaction with Zta is not directly affected by methylation. Class II ZREs contain a CpG motif; Zta interacts with them in the non-methylated state and displays somewhat enhanced binding to the methylated state [3]. Class III ZREs contain one or more CpG motifs and display null to poor binding by Zta in the non-methylated state but are recognized by Zta in the methylated state [28,29]. Thus class III ZREs are only recognized by Zta when methylated [28–31]. The three ZREs within Rp contain an example of each class of ZRE: RpZRE1 is class I; RpZRE2 is class II and RpZRE3 is class III.

Role for ZTA in overturning epigenetic silencing
The basic region of the bZIP domain of Zta contains a cysteine residue, Cys-189, which regulates the redox-sensitivity of DNA-binding activity [30]. Replacing this cysteine with a serine residue (ZtaC189S) is sufficient to prevent EBV genome replication [30,32]. Zta requires Cys-189 in order to activate Rp [31]. ZtaC189S is competent for interaction with
Table 1 | Three classes of ZREs

<table>
<thead>
<tr>
<th>Class I ZRE</th>
<th>Class II ZRE</th>
<th>Class III ZRE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contains CpG motif</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Binds non-methylated ZRE</td>
<td>N/A</td>
<td>Yes</td>
</tr>
<tr>
<td>Binds methylated ZRE</td>
<td>RpZRE1</td>
<td>RpZRE2</td>
</tr>
<tr>
<td>Example from Rp</td>
<td>RpZRE1</td>
<td>RpZRE2</td>
</tr>
<tr>
<td>Example sequences</td>
<td>TGGCTCA</td>
<td>TGGCTCA</td>
</tr>
</tbody>
</table>

chromatin-embedded RpZRE1 and RpZRE2 but defective for interaction with RpZRE3 in vivo [31]. This suggested that interaction with RpZRE3 is critical for activation of this promoter in vivo. Further investigation of the requirement for Cys-189 for interaction with RpZRE1–3 in vitro allowed the defect to be mapped to a fundamental deficiency in interacting with methylated RpZRE3 [31]. Therefore activation of Rp in B-cells correlates with the ability to bind to methylated RpZRE3, highlighting the direct role of Zta in overcoming epigenetic silencing of the viral genome. Intriguingly, in epithelial cells harbouring EBV, ZtaC189S is able to activate expression of Rp [30], suggesting that there are either lineage-specific differences in epigenetic silencing or in the local milieu of transcription factors between these two sites of viral replication.

Molecular interactions between ZTA and methyl cytosines

Kenney’s group has shown that single point mutations of Zta, at Ser-186, compromises the ability of Zta to bind the methylated but also to the non-methylated forms of ZREs, although this mutant is not helpful in differentiating between interactions with methylated and non-methylated sites [29]. Thus two residues are critical for the interaction with methylated ZRE: Cys-189 and Ser-186. The potential interactions of these two residues with methyl cytosines in the RpZRE2 and RpZRE3 were modelled from the known Zta bound to an AP1 site [21], generating a structural model of Zta bound to methylated RpZRE3 that reasonably accounts for a number of experimental observations [31]. In particular, the model rationalizes why the binding affinity of Zta for unmethylated RpZRE3 is far lower than for RpZRE2, why methylation increases the binding affinity for both sites, why the C189S mutation compromises methylated ZRE recognition and why the latter effect is more pronounced for RpZRE3 than for RpZRE2.

(i) The modelled ZRE structures account for the dramatically decreased ability of Zta to interact with non-methylated RpZRE3 compared with an AP1 site or RpZRE2. This centres on the interaction of Asn-182 with base-pairs ±2 in the AP1 site TGGCTCA; no equivalent contacts in RPZRE3 are predicted as the equivalent positions are T and G respectively (Table 1) [31].

(ii) The model explains the enhanced binding to methylated RpZRE2 and RpZRE3. RpZRE3 contains two adjacent CpG motifs. Methylation of CpG motif 1 (Table 1) results in a direct contact between the cytosine' methyl group and Ser-186Lef and a hydrophobic contact between Cys-189Left and the cytosine' methyl group (Figure 1) [31]. Thus both Ser-186Lef and Cys-189Left are postulated to interact with CpG motif 1 so as to simultaneously engage the two methyl groups located on complementary DNA strands (Figure 1). Methylation of CpG motif 2 is also found to influence Zta binding. The cytosine' interactions with Arg-190Left would be enhanced by methylation. On the complementary strand, the cytosine' methyl group contacts Ser-186Right [31]. Cys-189Right is too distant from any cytosine methyl group to form a direct contact, but it forms van der Waals contacts with the equivalent methyl group of thymidine' (Figure 1). Thus mutation of Cys-189 in ZtaC189S is predicted to destabilize contacts in both motifs of methylated RpZRE3, but only one
in methylated RpZRE2. This agrees neatly with the finding that the mutation more severely impairs Zta binding to methylated RpZRE3 than that to methylated RpZRE2 [31].

Cys-189 is implicated in the redox-sensitivity of Zta’s DNA-binding activity, and nitrosylation of this residue has been evoked as one possible mechanism by which nitric oxide down-regulates EBV re-activation [30]; such regulatory phenomena might potentially be linked to methylated ZRE recognition and Rp activation.

**Summary**

Thus, through the use of a mutant that is specifically deficient in binding to methylated RpZRE3, it has been established that methylated RpZRE3 recognition is essential for the disruption of latency in B-lymphocytes. This provides a mechanism by which an EBV gene can overturn the epigenetic silencing of viral genes that was imposed by the host cell. The potential for Zta to overturn epigenetic silencing of other viral genes and of host genes remains to be determined, as does the molecular mechanisms by which the interaction influences chromatin structure.

Methylation-enhanced affinity for DNA motifs is conceivably unique to Zta, as no other bZIP proteins are known to possess a serine residue equivalent to Ser-186. On the other hand, Cys-189 is relatively conserved among bZIP proteins: in a sequence alignment of 50 human bZIP proteins, over one-half conserve this residue [21]. It is therefore tempting to speculate that CpG methylation may enhance the affinity of certain cellular bZIP proteins for their cognate DNA target sites. This tantalizing potential for Zta’s cellular homologues to overturn the epigenetic silencing of genes awaits further investigation.

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**References**


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