MEF2-mediated recruitment of class II HDAC at the EBV immediate early gene *BZLF1* links latency and chromatin remodeling

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In B lymphocytes induced to proliferate *in vitro* by the Epstein–Barr virus (EBV), extra-chromosomal viral episomes packaged in chromatin persist in the nucleus, and there is no productive cycle. A switch from this latency to the productive cycle is observed after induced expression of the EBV *BZLF1* gene product, the transcription factor EB1. We present evidence that, during latency, proteins of the myocyte enhancer binding factor 2 (MEF2) family are bound to the *BZLF1* promoter and recruit class II histone deacetylases. Furthermore, we propose that latency is determined primarily by a specific and local recruitment of class II histone deacetylase (HDAC) by MEF2D to the *BZLF1* gene promoter. The switch from latency to the productive cycle could be due in part to post-translational modification of MEF2 proteins and changes in the local acetylation state of the chromatin.

INTRODUCTION

The Epstein–Barr virus (EBV) is a human γ -herpesvirus that infects, with life-long persistence, almost all of the adult population. EBV persistence is thought to be a major risk factor for several human cancers. In the peripheral blood, the site of persistence has been proposed to be resting memory B cells, in which there is no EBV gene expression, conforming to the definition of a 'true latency' (Babcock *et al.*, 1998). However, there is intermittent activation of replication and shedding of infectious virus *in vivo*. This activation has been associated with the emergence of human cancers, suggesting that the mechanism by which activation of the productive cycle occurs is a key determinant of EBV pathogenesis.

The molecular mechanisms involved in the switch from latency to activation of the productive cycle can be studied

in vitro using the model of EBV-infected B-lymphocyte cell lines. Indeed, EBV infects resting B lymphocytes and induces their proliferation. In such infected cells, a small subset of viral genes is expressed. The viral genome persists as an extra-chromosomal episome packaged in chromatin (Shaw *et al.*, 1979), and there is no productive cycle. The switch from latency to the productive cycle can be observed *in vitro* after treatment of EBV-infected cells with various agents, including phorbol esters associated with sodium butyrate (TPA/BA), calcium ionophores, transforming growth factor β (TGF- β) and anti-immunoglobulin antibodies (anti-IgG) (Faggioni *et al.*, 1986; Takada and Ono, 1989; Fahmi *et al.*, 2000). Through different pathways, these agents induce the expression of the two EBV transcription factors EB1 and R, products of the two EBV immediate early genes *BZLF1* and *BRLF1*, respectively. EB1 and R bind to specific sequences in the promoters of the EBV early genes and activate their transcription. They are also involved in the activation of the replication origin (OriLyt) used during the viral productive cycle and thus in the activation of the late genes (Feederle *et al.*, 2000).

The switch from latency to the productive cycle can also be obtained efficiently by transfecting an EB1 expression vector (but not an R expression vector) into EBV-infected B cells, suggesting that EB1 is the primary target for reactivation (Chevallier-Greco *et al.*, 1986). The regulation of EB1 expression is therefore likely to be a key determinant for maintenance of latency or for activation of the productive cycle. Treatment of infected cells with TPA plus sodium butyrate or trichostatine A, which are potent inhibitors of histone deacetylases (HDACs), induces the expression of EB1 and the productive cycle. Moreover, it has been proposed recently that changes in histone acetylation at the *BZLF1* promoter correlate with activation of expression of the

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BZLF1 gene (Jenkins *et al.*, 2000). Since targeted histone deacetylation/acetylation interplay has been associated with repression or activation of transcription, such modification of histones at the *BZLF1* promoter could be part of the mechanism by which latency and the productive cycle are regulated.

Interestingly, several binding sites for the myocyte enhancer binding factor 2 (MEF2) family of transcription factors have been characterized previously as *cis*-acting elements of the *BZLF1* promoter (Speck *et al.*, 1997). MEF2 proteins are known to interact with class II HDACs (HDAC4, HDAC5 and HDAC7) and this interaction results in targeted repression of transcription (Miska *et al.*, 1999; Lu *et al.*, 2000a,b). MEF2 proteins are activated by several kinases, including Erk5 and CaMK, that stimulate the MEF2 transcriptional activity by disrupting MEF2/HDAC complexes and allowing the recruitment of histone acetyl-transferases (HATs) (Lu *et al.*, 2000a). The MEF2 proteins are thus good candidates to target HDAC/HAT to the *BZLF1* promoter and then participate in the transcriptional regulation of the *BZLF1* gene. In the present study, we have investigated whether class II HDACs, especially HDAC4 and HDAC5, can influence expression of the EBV *BZLF1* gene.

RESULTS

Both VP16-HDAC4 and VP16-HDAC5 activate productive cycle genes in B cells latently infected with EBV

It has been shown previously that MEF2-D interacts, *in vitro*, with *cis*-regulatory elements on the *BZLF1* promoter (Figure 1A) (Liu *et al.*, 1997) and that class II HDACs such as HDAC4, HDAC5 and HDAC7, which contain specific domains mediating their interaction with MEF2, can be recruited to promoters via MEF2 proteins and thus repress transcription (Miska *et al.*, 1999; Lu *et al.*, 2000b). In order to evaluate whether HDAC4 or HDAC5 could be recruited to the *BZLF1* promoter by MEF2 proteins, we made use of plasmids expressing modified HDAC4 or HDAC5 proteins, converted from inhibitors to activators by fusion of their MEF2 binding domains to the herpes simplex virus VP16 activation domain (Figure 1B) and asked whether these constructions were able to activate EBV productive cycle genes in the EBV latently infected B cell line, Raji. These fusion proteins, called VP16-HDAC4 and VP16-HDAC5, respectively, are deleted of their catalytic domain and have been shown previously to be potent transcriptional activators when recruited to promoters by MEF2 proteins (Lu *et al.*, 2000b). The model generally accepted to explain the switch from latency to the productive cycle in EBV-positive B cells suggests that the inducers' primary target is the promoter of the *BZLF1* gene whose product induces expression of the *BRLF1* gene. Then EB1 and R activate the expression of all the early genes. Accordingly, after treatment of Raji cells with TPA/BA, we could easily detect, by western blotting, both EB1 and R proteins as well as a typical early gene product, the BMRF1 protein (or EA-D) (Figure 1C, lane 1), whereas expression of these proteins was not detectable in non-treated cells (Figure 1C, lane 2). When VP16-HDAC4 and VP16-HDAC5 proteins were transiently expressed in Raji cells, they both activated the expression of EB1 and consequently expression of R and BMRF1

Fig. 1. VP16-HDAC4/5 are specifically recruited at the *BZLF1* gene promoter *in vivo* and activate the switch from latency to the productive cycle. (A) Schematic representation of the *BZLF1* gene promoter. Binding sites for cellular (MEF2, Sp1/Sp3, CREB) and viral (ZRE: EB1 responsive element) proteins are represented along the promoter region by light-gray and dark-gray boxes, respectively. (B) Schematic representation of class II HDACs (HDAC4 and HDAC5) and their modified versions, VP16-HDAC4 and VP16-HDAC5. (C) Western blots from Raji cell extracts. The equivalent of 2×10^6 cells was loaded onto each lane of a 10% SDS-PAGE. Membranes were incubated with either the anti-BRLF1, the anti-BMRF1 or the anti-BZLF1 Mab. Lane 1, Raji cells treated with TPA/BA; lane 2, untreated Raji cells; lane 3, Raji cells transfected with VP16-HDAC4 expression plasmid; lane 4, Raji cells transfected with VP16-HDAC5 expression plasmid. (D) Left: the dark-gray boxes represent the EB1 responsive elements present on these promoters, and the light-gray boxes represent the MEF2 sites on the Zp promoter. Right: histogram of representative results from CAT ELISA made following co-transfection of DG75 cells with the reporter plasmids and the transactivator expression plasmids as indicated.

(Figure 1C, lanes 3 and 4). The VP16 activation domain alone had no effect (data not shown). These results suggest that the chimeric VP16-HDAC4 or VP16-HDAC5 proteins are recruited to the *BZLF1* promoter by a factor already present on the promoter.

The *BZLF1* promoter is a specific target for both VP16-HDAC4 and VP16-HDAC5

To demonstrate that VP16-HDAC4 and VP16-HDAC5 were specifically targeted to the *BZLF1* promoter but not to other EBV early gene promoters, EBV-negative B cells DG75 were transiently transfected with constructions containing either the *BZLF1* promoter or promoters of two well-characterized early genes, *BMLF1* and *BRRF1*, cloned upstream of the CAT reporter gene (Zp-CAT, Mp-CAT and Nap-CAT, respectively, Figure 1D). Since the *BZLF1*, *BMLF1* and *BRRF1* promoters contain EB1 binding sites, CAT gene expression was activated when Zp-CAT, Mp-CAT and Nap-CAT were co-transfected with an expression plasmid for EB1 (Figure 1D, compare lanes 2, 6 and 10 with 1, 5 and 9). However, both VP16-HDAC4 and VP16-HDAC5 increased the amount of CAT protein expressed from the Zp-CAT construct (Figure 1D, lanes 3 and 4), whereas they had no effect on the amount of CAT protein expressed from Mp-CAT or Nap-CAT (Figure 1D, lanes 7, 8, 11 and 12). These results demonstrate that the recruitment of VP16-HDAC4 and VP16-HDAC5 is specific for the *BZLF1* promoter. Since Mp-CAT and Nap-CAT do not contain MEF2 binding sites, these results strongly suggest that MEF2 may be involved in the specific recruitment of VP16-HDAC4 or VP16-HDAC5 to the *BZLF1* promoter.

Class II HDACs are specifically recruited on the *BZLF1* promoter by MEF2 proteins

In order to show that VP16-HDACs are indeed targeted to the *BZLF1* promoter via MEF2 proteins, we specifically mutated the three MEF2 binding sites of the Zp promoter, either individually or in combination, in the Zp-CAT construct. We also specifically mutated the Sp1/Sp3 binding site. DG75 cells were then co-transfected with the VP16-HDAC4 expression plasmid and the Zp-CAT reporter plasmid or the various mutated versions depicted in Figure 2B. The results presented show that the Sp1/Sp3 binding site does not contribute significantly to the VP16-HDAC4-mediated activation of transcription at the Zp promoter (compare lanes 4 and 1). In contrast, when one of the MEF2 binding sites was mutated, the VP16-HDAC4-mediated activation of transcription at the Zp promoter was significantly affected (lanes 2, 3 and 5): the two most upstream binding sites seem to contribute the most, since their mutation leads to ~50% loss of CAT expression (compare lanes 2 and 3 with lane 1), whereas the mutation of the proximal binding site affected the CAT expression level by only 20% (compare lanes 5 and 1). Mutation of the two upstream binding sites together leads to an 80% loss of CAT expression (compare lanes 6 and 1), and the simultaneous mutation of the three MEF2 binding sites almost completely abolishes the VP16-HDAC4-mediated activation of transcription at the Zp promoter (lane 7). Activation of transcription at the Zp promoter by VP16-HDAC4 is thus dependent on the presence of MEF2 binding sites.

In order to further demonstrate that the specific recruitment of HDAC4 onto the *BZLF1* promoter occurs via MEF2 proteins, we deleted the MEF2 interaction domain from VP16-HDAC4 (Figure 2A) and asked whether this mutated protein could still activate transcription at the *BZLF1* promoter. The

Fig. 2. MEF2 family proteins mediate the effect of class II HDAC on the *BZLF1* gene promoter. (A) Schematic representation of the HDAC4 protein and its modified versions, HDAC4 Δ MEF2, VP16-HDAC4 and VP16-HDAC4 Δ MEF2. The MEF2 interacting domain is shown in black and the deleted region is represented by a thin line. (B) Left: schematic representation of the Zp-CAT reporter constructs co-transfected with the VP16-HDAC4 expression plasmid. Right: histogram of the results of a representative CAT ELISA made following co-transfection of DG75 cells with the reporter constructs indicated on the left of the figure and an VP16-HDAC4 expression plasmid. (C) Histogram of the results of a representative CAT ELISA made following co-transfection of DG75 cells with the Zp-CAT reporter construct and expression plasmids for VP16-HDAC4 and VP16-HDAC4 Δ MEF2 as indicated. (D) Histogram of the results of a representative CAT ELISA made following co-transfection of DG75 cells with the reporter plasmids Zp-CAT or Nap-CAT and the expression plasmids (coding for EB1, HDAC4, HDAC4 Δ MEF2 or HDAC1) as indicated.

expression levels of the VP16-HDAC4 and VP16-HDAC4 Δ MEF2 proteins were similar, as determined by western blotting (data not shown). The results show that the VP16-HDAC4 Δ MEF2 protein is three times less efficient than VP16-HDAC4 in activating expression of CAT protein from the Zp-CAT construct (Figure 2C, compare lanes 3 and 2). These latter observations confirm that VP16-HDAC4 or VP16-HDAC5, and, by extension, HDAC4 and HDAC5, are specifically targeted to the *BZLF1* promoter via MEF2 proteins.

Overexpression of HDAC4 inhibits EB1-mediated activation of transcription only at the *BZLF1* promoter

If the repressive effect of HDACs on transcription is only seen when they are locally recruited to chromatin by DNA-binding transcription factors, then overexpression of HDAC4 should only repress EB1-mediated activation of promoters carrying MEF2 binding sites. In order to test this hypothesis, the Zp-CAT or Nap-CAT reporter constructs were co-transfected in DG75 cells together with expression plasmids for EB1 or EB1 plus HDAC4. In these experiments, HDAC4 repressed four times the EB1-mediated activation of Zp-CAT transcription (Figure 2D, compare lanes 3 and 2), whereas it had no effect on EB1-mediated activation of Nap-CAT (Figure 2D, compare lanes 8 and 7). HDAC4 Δ MEF2 (Figure 2A), which does not interact with MEF2, was unable to repress the EB1-mediated activation of transcription from Zp-CAT (Figure 2D, lane 4). As expected, HDAC4 Δ MEF2, like HDAC4, had no effect on Nap-CAT activity (Figure 2D, lane 9). The expression levels of the HDAC4 and HDAC4 Δ MEF2 proteins were similar, as determined by western blotting (data not shown). HDAC4 can thus inhibit EB1-mediated activation of transcription at promoters containing MEF2 binding sites. This effect is specific for class II HDACs, since HDAC1 has only a very weak effect on EB1-mediated activation of Zp-CAT transcription (Figure 2D, lane 5). These results corroborate the hypothesis that specific recruitment of HDACs to the *BZLF1* promoter, and, by extension, local deacetylation of histones, down-regulates *BZLF1* gene expression and participates in latency.

The *BZLF1* promoter is subjected to chromatin acetylation modifications after activation of the productive cycle

In order to determine whether the acetylation state of histones at the Zp promoter was indeed altered during activation of the productive cycle in EBV-infected Akata cells, we performed chromatin immunoprecipitation assays. Soluble chromatin was prepared from Akata cells untreated or treated with anti-IgG to induce *BZLF1* gene expression and consequently the productive cycle. The cross-linked chromatin was then immunoprecipitated with antibodies specific for acetylated histone H3 or H4. The immunoprecipitated DNA was analyzed by PCR using primers designed to amplify sequences spanning the Mp, Cp and Zp promoters. As shown in Figure 3A, the amount of acetylated histone H3 and H4 found on Mp and Zp was significantly higher in the anti-IgG-treated cells (lanes 2, 6, 8 and 12) compared with the untreated cells (lanes 1, 5, 7 and 11). In contrast, anti-IgG treatment made no difference to the amount of acetylated histone H3 and H4 on the Cp promoter (compare lanes 4 and 10 with 3 and 9), which is in agreement with the observation that this promoter is inactive in uninduced Akata cells and not activated during the EBV productive cycle (Takada and Ono, 1989). These data support the notion that transcriptional repression of the *BZLF1* gene is linked to histone deacetylation and that histone acetylation of the chromatin at the Zp promoter occurs *in vivo* following activation of the productive cycle.

Fig. 3. (A) Effect of the induction of the EBV productive cycle in Akata cells upon acetylation at the Zp promoter. H3 and H4 acetylation of the Zp, Cp and Mp promoters was determined by CHIP assay using anti-acetylated H3 or anti-acetylated H4 antibodies in untreated Akata cells and in Akata cells treated for 3 h with anti-IgG. (B) Model of transcriptional regulation at the *BZLF1* gene promoter. During latency, MEF2-D proteins are present on the *BZLF1* gene promoter and they mediate local histone deacetylation on this promoter by recruiting class II HDAC. This contributes to the absence of expression of the *BZLF1* gene and maintenance of latency. After reactivation of the productive cycle by different inducers, the MEF2-D proteins are modified so that they do not interact with class II HDAC but do interact with HATs and/or other cellular factors such as NFAT.

DISCUSSION

In this paper, we present new aspects of EBV gene expression regulation that link latency in EBV-infected B cells to chromatin remodeling. We propose that class II HDACs such as HDAC4 and HDAC5 inhibit expression of the EBV *BZLF1* gene, whose product is a transcription factor regulating the switch from latency to the productive cycle. This inhibition appears to be mediated by specific recruitment of HDAC proteins to the *BZLF1* gene promoter via DNA-bound MEF2 proteins. The MEF2 factor involved is probably MEF2-D, as it was shown (Liu *et al.*, 1997) that, in EMSA experiments using extracts from B cells and specific antibodies, only MEF2-D could be identified in complexes that bound the MEF2 elements of the *BZLF1*

promoter. Furthermore, MEF2-D has previously been shown to interact directly with HDAC4 (Choi *et al.*, 2001).

Our results support a model in which activation of the EBV *BZLF1* gene is dictated by a balance of positive and negative effects on the transcriptional activity of MEF2 family proteins. Accordingly, the MEF2 binding sites present on the *BZLF1* promoter are the most important contributors to the activity of this promoter in response to signal transduction from anti-IgG or TPA/BA (Flemington and Speck, 1990; Jenkins *et al.*, 2000), suggesting that these sites are involved in the targeting of proteins with a major role in transcriptional activation and/or chromatin modification. This is consistent with the now well-documented model that class II HDACs repress transcription by deacetylating core histones associated with regulatory regions controlled by MEF2 family proteins. Post-transcriptional modifications of chromatin regulate the access of transcription factors to regulatory DNA sequences and consequently gene expression. The deacetylation of lysine residues in the core histones N-terminal tails is one of the chromatin modifications that correlates with transcriptional inhibition. On the other hand, there are many examples of transcriptional activators that have been shown to act by recruiting proteins with histone acetylase activity to specific promoters (Strahl and Allis, 2000).

Different signal transduction pathways are involved in the transition from latency to productive viral replication. It is now accepted that the mechanisms of reactivation from latency to the productive cycle imply activation of phospholipase C, which subsequently increases intracellular Ca^{2+} , which in turn induces activation of the calcium/calmodulin-dependent kinase type IV/Gr and the calcium/calmodulin-dependent phosphatase calcineurin. This leads to modification of the MEF2 family proteins and associated factors. Indeed, it has been shown that post-translational modifications of a MEF2 family member by the p38 MAP kinase can activate MEF2-dependent gene expression (Yang *et al.*, 1999; Zhao *et al.*, 1999). These post-translational modifications have been shown to destabilize the HDAC4-MEF2 interaction and to contribute to relocalization of HDAC4 or HDAC5 from the nucleus to the cytoplasm. In addition, activated MEF2 proteins can interact with p300, which has HAT activity (Chatila *et al.*, 1997; Liu *et al.*, 1997; Sartorelli *et al.*, 1997; Corcoran and Means, 2001). MEF2-responsive elements on the *BZLF1* promoter are thus direct targets of this pathway. In conclusion, our results lead us to propose a model in which MEF2 family transcription factors are permanently recruited on the *BZLF1* gene promoter (Figure 3B). During latency, MEF2 acts as a repressor by recruiting a class II HDAC. After treatment of the cells with an inducer, MEF2 is modified and recruits HATs such as CBP or p300 as well as other activators like NFAT or Smad2 (Blaeser *et al.*, 2000; Corcoran and Means, 2001; Quinn *et al.*, 2001), and consequently MEF2 becomes a strong activator of *BZLF1* gene expression.

METHODS

Cell culture. Raji (EBV-infected), Akata (EBV-infected) and DG75 (non-infected) B cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. Induction of the productive cycle was obtained by treating the Raji cells with TPA (20 ng/ml) associated with BA (3 mM) for 48 h or the Akata cells with 0.1 μ g/ml anti-human IgG for 3 h.

Plasmids and transfections. Expression vectors for EB1 and the reporter plasmids Zp-CAT, Mp-CAT and Nap-CAT have been described elsewhere (Le Roux *et al.*, 1996; Segouffin-Cariou *et al.*, 2000). The Zp(MA)-CAT, Zp(MB)-CAT, Zp(MC)-CAT, Zp(MD)-CAT, Zp(MA/B)-CAT and Zp(MA/B/C)-CAT reporter plasmids were mutated versions of Zp-CAT in which the MEF2 sites, the Sp1/Sp3 site or their different combinations have been changed from TATTTT to TAGGGTT (MEF2 A site), from TTATTTT to TTCGGTT (MEF2 B site), from TCTTTTA to TCGGGTA (MEF2 C site) and from TAAATTTA to TACCGTTA (Sp1/Sp3 site D), using the QuickChange Site-Directed Mutagenesis kit (Stratagene). The HDAC4 and VP16-HDAC4/5 expression plasmids were kindly provided by T. Kouzarides (Miska *et al.*, 1999) and E. Olson (Lu *et al.*, 2000b), respectively. HDAC4 $_{\Delta$ MEF2 is a HDAC4 deletion mutant lacking amino acids 163–180 located in the MEF2 binding domain and generated by using the QuickChange Site-Directed Mutagenesis kit. Transfection of B cells (10^7 cells) was carried out with 20 μ g of each plasmid DNA, using electroporation (220 V and 950 μ F with a Bio-Rad GenePulser). CAT expression was monitored 48 h after transfection by CAT ELISA, using 1% of the cell extract, according to the manufacturer's specifications (Roche). Each transfection was conducted several times and a representative result is shown for each experiment.

Western blotting. Cells were lysed in SDS-gel sample buffer. The equivalent of 2×10^6 cells was loaded into each lane of SDS–10% polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose membranes. Membranes were incubated with either the anti-BZLF1 Mab Z125, the anti-BRLF1 Mab 8C12 (Le Roux *et al.*, 1996) or the anti-BMRF1 Mab (NCL-EADE31, Novocastra). Rabbit anti-mouse horseradish peroxidase conjugate (Amersham) was used as secondary antibody, and immune complexes were visualized using ECL (Amersham).

Chromatin immunoprecipitation (CHIP) assay. The method used was adapted from Orlando *et al.* (1997). Chromatin was prepared from 10^6 Akata cells (treated or untreated with anti-IgG) fixed by the addition of formaldehyde (1%) and sonicated such that the average DNA fragment length was reduced to <1 kbp. One percent of the chromatin solution (input chromatin) was kept to quantify the amount of immunoprecipitated DNA. Immunoprecipitation was performed in 1.2 mM EDTA, 0.01% SDS, 1.1% Triton X-100, 167 mM NaCl, 16.7 mM Tris–Cl pH 8.0 using polyclonal antibodies directed against acetylated histone H4 (Upstate Biotechnology; catalog number 06-866) or acetylated histone H3 (Upstate Biotechnology; catalog number 06-599). Protein–DNA cross-links from the inputs and immunoprecipitates were reversed by heating at 65°C for 4 h and the DNA fragments purified (proteinase K digestion, phenol–chloroform extraction and ethanol precipitation). Purified DNA from the inputs and immunoprecipitation assays was used for PCR amplification. The PCR reaction was conducted in non-saturating conditions in order to quantify the amount of immunoprecipitated DNA in comparison to a PCR made using a range of dilutions of the input DNA. The primers used were pZF19 (5'-TGGGCTGTCTATTTTACACAG-3') and pZB20 (5'-CTTCAGCAAAGATAGCAAAGGTGG-3') for the Zp promoter region, pMF9 (5'-AACCTCTTACATCACTCACTGCACG-3') and pMB8 (5'-GGGAATTTGTCTTCTGGCACAG-3') for the Mp promoter region and CpF53 (5'-AACCTTGTGGCGGAGAGAAG-3') and CpB61 (5'-GGCGAATTAAGTGAAGCTTGCG-3') for the Cp

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promoter region. Quantification of the immunoprecipitated DNA was made with the help of a Phosphorimager.

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