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Acetylation changes at lysine 5 of histone H4 associated with lytic gene promoters during reactivation of Kaposi′s sarcoma-associated herpesvirus

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Summary. – Kaposi′s sarcoma-associated herpesvirus (KSHV) is a pathogenic agent of Kaposi's sarcoma, primary effusion lymphoma and multicentric Castleman's disease in humans. Similarly to other gammaherpesviruses such as Epstein-Barr virus (EBV) and herpesvirus saimiri (HVS), KSHV displays two alternative life cycles, latent and lytic one. The transactivation from latency to the lytic phase is the result of transcriptional changes in the KSHV genome caused by the replication and transcriptional activator (RTA). During KSHV reactivation, epigenetic modifications of histone protein on the viral genome occur, which regulate the transcriptional activation of a number of lytic genes. The reactivation of EBV from latency to lytic cycle, induced by an immediate-early Zta protein, was shown to be accompanied by acetylation of specific lysines in histone H4. Accordingly, we hypothesized that the RTA-induced transactivation of KSHV could also be accompanied by histone acetylation. To validate this hypothesis, we assayed alterations of acetyl-histone H4-lysine 5 (acH4K5) during the RTA-mediated KSHV reactivation. While the modified histone protein in a total cell lysate was not distinguished between control and RTA-expressed cells, upregulated acH4K5 was detected on several lytic gene promoter regions during KSHV reactivation. Our results clearly indicate that this epigenetic change is related to transcription of genes expressed in the lytic cycle of KSHV.

Keywords: Kaposi′s sarcoma-associated herpesvirus; lytic reactivation; replication and transcription activator; histone H4; lysine acetylation

Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), is a human gammaherpesvirus discovered in 1995. KSHV plays an important role in the development of Kaposi′s sarcoma, the most common malignancy in AIDS patients. Additionally, KSHV is associated with lymphoproliferative disorders, such as primary effusion lymphoma and multicentric Castleman's disease. Similar to other gammaherpesviruses, including EBV and HVS, KSHV has two distinct and alternative life cycles, referred to as latent and lytic replication (Cesarman *et al*., 1995; Soulier *et al*., 1995). During the lytic replication, the KSHV replication and transcription activator (RTA) stimulates the expression of a number of viral genes, including K-bZIP encoded by ORF K8 of KSHV (Liao *et al*., 2003; West and Wood, 2003; Ganem, 2007).

Chromatin is a highly dynamic structure of nucleosomes composed of DNA wrapped around the core histones (H2A, H2B, H3, H4). These structures impart protection from damage and regulate genomic processes, including transcription and replication activities, as well as molecular mechanisms of quiescence, such as heterochromatic silencing (Kouzarides, 2007).

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Abbreviations: acH4K5 = acetyl-histone H4-lysine 5; CBP = CREB-binding protein; ChIP = chromatin immunoprecipitation; Dox = doxycycline; EBV = Epstein-Barr virus; HAT = histone acetyltransferase; HVS = herpesvirus saimiri; KSHV = Kaposi′s sarcoma-associated herpesvirus; LANA = latency-associated nuclear antigen; OriL = origin of lytic replication; PMSF = phenylmethylsulfonyl fluoride; qRT-PCR = quantitative real-time PCR

Histones are subjected to various posttranslational modifications (acetylation, methylation, phosphorylation, ubiquitination, SUMOylation, biotinylation, and ADP-ribosylation) capable of modulating chromatin structures to influence gene expression (Kouzarides, 2007). Histone acetylation is thought to be a prerequisite for transcriptional activation of genes that may undergo hereditary silencing. Transcriptional co-activators with intrinsic histone acetyltransferase (HAT) activity can be recruited by sequence-specific DNA-binding proteins to alter local histone acetylation pattern and stimulate transcription complex assembly. This mechanism remains poorly understood, since it is not clear how initial sequence recognition occurs in higher-order chromatin structures, which serve as a barrier limiting DNA accessibility (Utley *et al*., 1998; Hassan *et al*., 2001; Berger, 2002).

Similar to RTA, EBV immediate-early protein, Zta, is a transcription and DNA replication factor required to initiate lytic replication. The majority of viral genes required for lytic replication are repressed, but can be reactivated via Zta overexpression or treatment of cells with pleiotropic agents. Chromatin-based repression is suggested to be an important component in maintaining transcriptional silence of lytic cycle gene products during viral latency. This hypothesis is consistent with the finding that transcriptional co-activators with histone acetylase activity cooperate with Zta to stimulate lytic gene expression (Miller, 1989; Adamson and Kenney, 1999; Zerby *et al*., 1999).

Zta stimulates acetylation of all lysine residues in histones H3 and H4. Western blot analysis with acetyl-lysine-specific antibodies revealed that Zta targets specific lysine residues for acetylation by cAMP response element-binding protein (CREB)-binding protein (CBP). In particular, acetylation of lysines 5 and 12 of histone H4 is significantly increased after the addition of Zta (Deng *et al*., 2003). With regard to the well-known similarity of KSHV to EBV, in this study we investigated the possibility that RTA of KSHV also stimulates acetylation of specific lysines in histone H4 associated with KSHV lytic promoters. The results obtained by ChIP fully confirmed this assumption for the histone H4 lysine at position 5.

Materials and Methods

Cells. TREx BCBL-1 and TREx BCBL-1/RTA cells were grown in RPMI 1640 medium supplemented with 10% FBS at 37°C. Tetracycline-inducible RTA expression in TREx BCBL-1 cells has been described previously (Nakamura *et al*., 2003). To induce expression of RTA in TREx BCBL-1/RTA cells, cells were treated with 1 μg/ml doxycycline (Dox) and harvested at each time-point.

Western blot analysis. In total, $4x10^6$ cells were lysed in EBC buffer (10 mmol/l Tris-HCl; pH 7.5, 600 mmol/l NaCl, 0.5% Nonidet P-40) containing protease inhibitors (1 mmol/l phenylmethylsulfonyl fluoride (PMSF), 0.2 mmol/l Na_3VO_4). Cell lysates were resolved on 10% or 15% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. After blocking in 5% skim milk dissolved in TBST (Tris-buffered saline, 0.1% Tween 20) for 1 h, membranes were incubated with the indicated antibodies. Following antibody binding, membranes were washed five times with TBST buffer and subjected to enhanced chemiluminescence assays using rabbit or mouse IgG-horseradish peroxidase and SuperSignal substrate (Thermo Scientific) after a final wash. Anti-β-actin and anti-acetyl-histone H4-lysine K5 antibodies were purchased from Santa Cruz, and the anti-histone H4 antibody from Millipore. Anti-KbZIP antibody was purified from rabbit.

Chromatin immunoprecipitation (ChIP) assay. ChIP assay was performed according to the manufacturer's instructions (Millipore). Briefly, a total of 2×10^7 cells were cross-linked with 1% formaldehyde for 10 min at 37°C. After terminating the cross-linking reaction by 125 mmol/l glycine solution, the cells were washed with PBS containing 0.1 mmol/l PMSF and 1 μg/μl Leupeptin and resuspended in 600 μl SDS lysis buffer. The cells were sonicated (Bio-ruptor) to shear DNA into 500 bp fragments, and the sonicated samples were separated by centrifugation at 15,000xg for 10 min at 4°C. Supernatant fractions were diluted with dilution buffer and incubated with the indicated antibodies at 4°C for 16 hr. Protein A/G–agarose beads were added, followed by incubation at 4°C for 2 hr. The mixture was washed once with low-salt immune complex wash buffer, once with high-salt immune complex wash buffer, once with LiCl immune complex wash buffer and twice with TE buffer, and subsequently eluted in elution buffer (1% SDS, 0.1 mol/l $NaHCO_3$). The crosslinking of the DNA complexes was reversed by the addition of 200 mmol/l NaCl and incubation at 65°C for 16 h. DNA was subsequently treated with 2 μl proteinase K (10 μg/μl) at 45°C for 1 hr, collected using the PCR purification kit (QIAGEN) and subjected to real-time PCR. H4 and acetyl-H4-K5 antibodies were used for analyses.

Quantitative real-time PCR (qRT-PCR) analysis. qRT-PCR was performed using KAPA SYBR fast qPCR master mix (KAPA Biosystems) with specific primers (0.25 μmol/l) and templates of the experimental and control groups in a total volume of 20 μl. Standard curves representing 8-point serial dilutions of the template in the experimental and control groups were analyzed and used to calibrate the relative quantity of the product generated in the exponential phase of the amplification curve. The qRT-PCR protocol comprised one cycle at 95°C for 3 min, followed by 60 cycles with two steps each at 95°C for 3 sec and 60°C or 62°C for 20 sec. Real-time PCR data were obtained for at least three independent experiments. Normalized data were obtained by subtraction of the mock (no antibody) value from the experimental value and correction for the signal from input DNA. To measure the relative enrichment of the same gene in different cells, the values for histone modification were further normalized against that for histone H4 under individual experimental conditions. Normalized data were

obtained with three steps. (1) *β-actin* gene was used to adjust initial DNA concentration of each sample. (2) Data obtained from samples immunoprecipitated with H4 antibodies were adjusted to normalized DNA concentration. (3) Adjusted histone H4 data were used for normalization of acetyl-H4-K5-immunoprecipitated DNA concentrations (Ng *et al*., 2006).

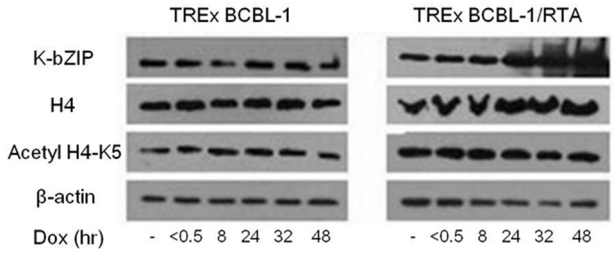

Fig. 1
Effect of RTA-induced reactivation of KSHV on acetylation of lysines in histone H4 associated with both viral and cellular genomes
Western blot analysis of TREx BCBL-1 and TREx BCBL-1/RTA cells untreated or treated with Dox for 0.5, 8, 24, 32, and 48 hr for K-bZIP, H4 and acetyl-H4-K5. β-actin served as control.

Table 1. Primers used in qRT-PCR

Promoter and coding region	Primer (5′ to 3′)
RTA promoter (-0.25 kb)	ATCTCCAATACCCGGAATT (F) TTTTGTGGCTGCCTGGA (R)
RTA coding region (+0.7 kb)	GAGAAACGCCGGCCAATT (F) GGGTTTGCTAATGACAAACT (R)
Origin of lytic replication (Left end)	CATGGGGTTGGGATTTTT (F) AATGGGCGTAACCGTAG (R)
PAN RNA promoter (-0.45 kb)	CACGTCCGCCAGCGAT (F) ATAAACTTTCATTAATGTTCAT (R)
PAN RNA promoter (-0.25 kb)	TAGTGATTCGGTAGATTTG (F) GCTAAACTGACTCAAGCT (R)
ORF57 promoter (-0.45 kb)	GGTCGTTCAATAGAGGAC (F) TGACTTCGCTCACCCTAA (R)
ORF57 promoter (-0.25 kb)	AACAGTCCGTGTATTCCTT (F) GTCCTTTGGTTCTTATATTG (R)
LANA promoter (-0.45 kb)	TTAGGTTCTAGGTTGTATTC (F) TCCAGGCTCTACAGGTAG (R)
LANA promoter (0.25 kb)	AGTTGCCATATAGAGTGGC (F) AAGCCACACCTCTCCCC (R)
LANA coding region (TSS)	ATGTCATTTCCTGTGGAGAGTCC (F) GCCCATAACTTATTGTGT (R)
Beta-actin coding region	CTGGAACGGTGAAGGTGACA (F) AAGGGACTTCCTGTAACAATG (R)
MDM2 coding region	ACTATTCTCAGCCATCAACT (F) CTTCCCTTTCAAACTCTTTCA (R)

The kb values represent KSHV genomic regions relative to the translational start site of indicated viral genes. TSS = transcriptional start site.

Results

Intercellular level of acetylated histone H4 at lysine 5 is not affected by RTA expression

Previous study has shown that the EBV-encoded lytic activator, Zta, enhances the acetylation of histones of oligonucleosomes via recruitment of CBP. In particular, acetylation at lysine residues K5, K8 and K12 of histone H4 is significantly increased after the addition of Zta *in vitro* (Deng *et al*., 2003). Taking into account that EBV is a well-known gammaherpesvirus homologous to KSHV, it is predicted that lysine residues of the histone on the KSHV genome are acetylated via expression of RTA.

In contrast to previous studies, we identified histone modifications via lytic reactivation using western blot analysis. To assess the ability of RTA in the tetracycline-inducible expression system to activate expression of other viral genes, such as immediate-early lytic protein K-bZIP (Lin *et al*., 1999), TREx BCBL-1 and TREx BCBL-1/RTA cells were treated with Dox for indicated times. As shown in Fig. 1, increased K-bZIP protein expression was detected in TREx BCBL-1/RTA cells after 24 hr Dox-treatment. This result indicates that Dox-induced RTA in this system leads to the expression of viral lytic gene in a well-ordered, kinetically appropriate manner. However, the quantities of histone H4 and acH4K5 were fixed, since the western blot analysis was performed using whole cell lysate, and histone proteins were not on specific viral genomes but on host and viral genomes. These results show that while KSHV lytic reactivation occurred via Dox-induced RTA expression in TREx BCBL-1/RTA cells, expressed RTA does not regulate the amount of histone H4 or the acetylated state of that on the intracellular level.

Acetylation changes at lysine 5 of histone H4 on the KSHV lytic gene promoters

A previous genome-wide ChIP analysis of the KSHV genome during latent and lytic reactivation focused on the modification of histone H3, but not H4 (Toth *et al*., 2010). We, therefore, performed ChIP assay to detect histone H4 epigenetically modified by RTA on the viral lytic gene promoter only.

Subsequently, qRT-PCR analysis was employed to assess the extent of variation in histones at the origin of lytic replication (OriL) region and RTA, ORF57, polyadenylated nuclear RNA (PAN RNA) and latency-associated nuclear antigen (LANA) putative promoters (Malik *et al*., 2004; Sun *et al*., 1996). Since the gene promoter regions were not clear, the qRT-PCR assay was performed with several potential promoter regions of each gene. As expected, we detected

increased acH4K5 on putative promoters of lytic genes and on the OriL region in cells displaying lytic reactivation promoted by Dox-inducible RTA expression. In contrast to lytic reactivated cells, acH4K5 levels on the lytic gene promoters were slightly decreased in TREx BCBL-1 cells. Although LANA is a latent protein, modification of histones on its putative gene promoters was increased to some extent (Fig. 2). These results clearly show that RTA expression regulates epigenetic modifications of histone protein to activate viral lytic gene expression during KSHV reactivation.

Discussion

Earlier studies have documented that epigenetic histone modification, in particular acetylation, plays a key role in the mechanism of reactivation of herpesviruses. For instance, Zta of EBV activates CBP HAT and recruits more complexes that are required for activation of EBV lytic transcription. The group additionally showed that the basic region of Zta is essential for targeting CBP to oligonucleosomes, which correlates with transactivation. Furthermore, ChIP analysis revealed that stable promoter-bound CBP and histone acetylation are dependent on the CBP bromodomain (Deng *et al.*, 2003). Since RTA of KSHV is highly homologous to Zta, we speculate that RTA has affinity for the CBP bromodomain.

Bromodomains of several different proteins are proposed to interact with acetylated lysines of histones via binding of four-helix bundles. The data suggest that bromodomains facilitate targeting of multiple lysine residues in the amino-terminal tails of histones. Bromodomain association with acetylated lysines may aid in targeting adjacent lysine residues on neighboring histone tails (Jacobson *et al.*, 2000; Zeng and Zhou, 2002).

If epigenetic histone acetylation is shown to be related to CBP HAT activity and its bromodomain, the underlying mechanism of epigenetic histone acetylation and significance of the bromodomain as a lysine residue target of histone proteins will be investigated in further detail.

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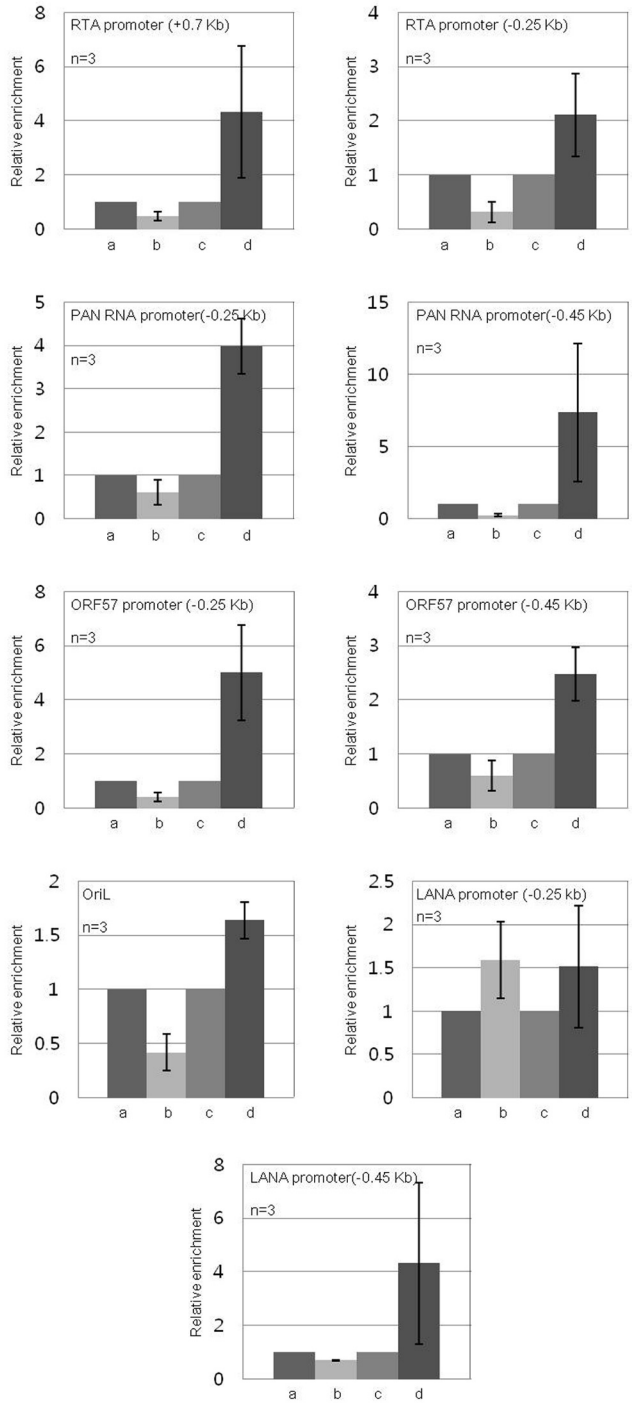

Fig. 2
Effect of RTA-induced reactivation of KSHV on acetylation of lysines in histone H4 associated with viral lytic gene promoters
TREx BCBL-1/RTA and TREx BCBL-1 cells treated with Dox for 12 hr were subjected to ChIP using the acetyl-H4-K5 antibody. Relative enrichment of acetyl-H4-K5 was assayed by qRT-PCR (for details see *Materials and Methods*). Individual figure parts correspond to specific promoters and OriL, respectively, with which was the histone H4 associated. a, TREx BCBL-1 Dox -; b, TREx BCBL-1 Dox +; c, TREx BCBL-1/RTA Dox -; d, TREx BCBL-1/RTA Dox +.

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