原やHBe抗原の有無では両群間に有意差はなかったが、HBe抗体陰性の患者の方が陽性の患者に比してMDSC頻度が高い傾向があった(p=0.051)。またHBV DNA 4 log copy/ml以上の患者の方が、4 log copy/ml未満の患者に比してMDSC頻度が有意に高かった。3 log copy/ml未満の患者は全例核酸アナログ製剤使用中のB型肝炎患者であった。

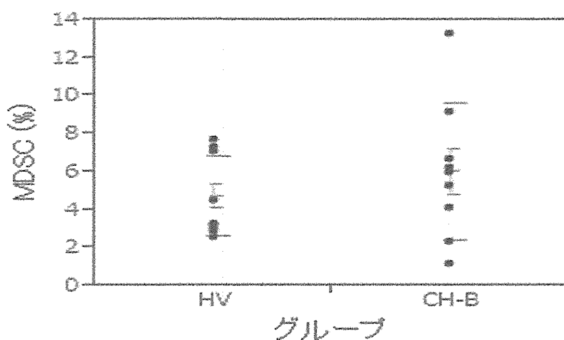

図2 B型慢性肝炎と健康成人におけるMDSCの頻度の比較

D. 考察

フローサイトメトリーにて$CD33^{+}CD11b^{+}CD14^{+}HLA\text{-}DR^{-}$分画のMDSCを同定することが可能であった。B型慢性肝炎患者の解析では、B型肝炎の病態によりMDSCの頻度が変化し、このことが病態形成に関与することが示唆された。特にセロコンバージョンやHBV DNA量と核酸アナログ製剤使用とMDSCの関係が示唆された。しかしながら現在のところ少数例の検討のため、十分な解析ができていない。今後さらに層別解析が可能な症例数まで検討していく必要があると共に、肝硬変症例、肝細胞癌合併症例も解析する必要がある。さらにMDSCの機能解析も合わせて行うことで、B型肝炎の病態形成におけるMDSCの意義を明らかにし、新規治療戦略の構築を試みたい。

E. 結論

B型慢性肝炎の病態形成にMDSCの関与が示唆された。

F. 研究発表

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2.学会発表

本年は本研究に基づく学会発表はなし

G.知的所有権の出願・取得状況

1.特許取得

なし

2.実用新案登録

なし

3.その他

なし

厚生労働科学研究費補助金（B 型肝炎創薬実用化等研究事業）
分担研究報告書

B 型肝炎におけるトリプトファン代謝酵素の免疫修飾作用と抗ウイルス作用の解析

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研究要旨：B 型慢性肝炎患者では宿主免疫系が撹乱されているが、その詳細な機序に関しては明らかではない。HBV による免疫修飾の責任分子が同定できれば、それを標的とした創薬への展望が得られる。Indoleamine-2,3-dioxygenase（IDO）はトリプトファンをキヌレニンに代謝し､免疫抑制作用を発揮する。種々の癌や難治性ウイルス感染症において、IDO の発現や活性の亢進が病態に関与することが報告されている。本年度は、HBV 感染症における IDO 活性と、その誘導機序や免疫学的機能を検討し、治療標的としての可能性を明らかにすることを目的とした。HBV 陽性患者では、血清キヌレニンは非感染者よりも高値であった。血清キヌレニン値は慢性肝炎、肝硬変、肝癌の群間では差を認めず、血清 ALT 値、HBVDNA 量とも相関を認めなかった。HBV 複製肝癌細胞（HepG2.2.15、HB611）における IDO 活性は、HBV 陰性細胞と比べて差を認めなかったが、IFN-γ などの炎症性サイトカインによって、HBV 複製肝癌細胞において誘導された。以上より、HBV 感染により患者 IDO 活性は亢進しているが、HBV 感染のみでは有意な IDO 活性は得られず、炎症の関与が必要であることが示唆された。IDO の治療標的としての有用性に関しては、今後の検討が必要である。

A. 研究目的

B 型慢性肝炎患者では宿主免疫系が撹乱されているが、その詳細な機序に関しては明らかではない。HBV による免疫修飾の責任分子が同定できれば、それを標的とした創薬への展望が得られる。Indoleamine-2,3-dioxygenase（IDO）はトリプトファンをキヌレニンに代謝し、免疫抑制作用を発揮する。種々の癌や難治性ウイルス感染症において、IDO の発現や活性の亢進が病態に関与することが報告されている。また､IDO は IFN-γ で誘導される ISG の側面も持っており､HBV 複製肝癌細胞に発現すると､HBV 複製を抑制することが報告されている。本年度は、HBV 感染症における IDO 活性と、その誘導機序を検討し、免疫修飾作用や抗 HBV 作用を介した治療標的としての可能性を明らかにすることを目的とした。

B. 研究方法

B 型肝炎患者（慢性肝炎、肝硬変、肝癌）と非感染健康成人から血清を採取し、血清キヌレニン（Kyn）を HPLC 法で測定し、Systemic IDO 活性の指標とした。肝炎患者の臨床マーカー（ALT 値、血小板数、HBVDNA 量、肝組織所見など）と Kyn 値との相関の有無を検討した。HBV 複製を維持する HepG2.2.15 または HB611 の培養上清中の Kyn 値を、それぞれの親株 HepG2、Huh6 の Kyn 値と比較した。また、IFN-γ、LPS などを添加した際の Kyn 値も評価した。

（倫理面への配慮）

本研究は大阪大学医学部倫理委員会の承認を受けており、事前に被験者の同意を得ており倫理的問題はないと考える

C. 研究結果

B 型肝炎患者では、血清 Kyn 値は非感染者よりも有意に高値であった。血清 Kyn 値は慢性肝炎、肝硬変、肝癌の群間では差を認めず、血清 ALT 値、HBVDNA 量とも相関を認めなかった。また肝組織の F 因子、A 因子との相関も認められなかった。HBV 複製肝癌細胞（HepG2.2.15、HB611）における上清中 Kyn 値は、HBV 陰性細胞と比べて差を認めなかったが、IFN-γ、LPS 存在下では、HBV 複製細胞においてのみ、上清中 Kyn 値が増加した。

D. 考察

B 型慢性肝炎患者では、Systemic IDO 活性は亢進しているが、臨床マーカーとの関連性は明らかではなかった。培養細胞での検討からも、HBV 感染のみでは有意な IDO 活性は得られず、炎症などの関与が必要であることが示唆された。

E. 結論

B 型肝炎患者では、HBV 感染によって Systemic IDO 活性が亢進しているが、その治療標的としての有用性に関しては、今後の検討が必要である。

F. 研究発表

1. 論文発表

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G. 知的所有権の出願・取得状況

1. 特許取得
なし
2. 実用新案登録
なし
3. その他
なし

厚生労働科学研究費補助金（B型肝炎創薬実用化等研究事業）
分担研究報告書

HBVポリメラーゼの発現・精製と活性測定系の確立

研究分担者　大崎　恵理子　大阪大学大学院医学系研究科　助教

研究要旨：現在HBV感染症に使用されている抗HBV剤は抗HIV剤の流用であり、HBVの本質を理解した真の抗HBV剤は開発されていない。HBVポリメラーゼ（HBVpol）の特性に基づいた、或は立体構造に根ざした迅速な抗HBV開発システムの構築が望まれる。今回、HBVpol（末端蛋白［terminal protein; TP］-スペーサー領域［spacer region; SP］-逆転写酵素ドメイン［reverse transcriptase; RT］-RNaseHドメイン［RNaseH; RNH］のうち、TP及びRTドメインについて、GST（glutathione S transferase）タグ付加発現系を構築し大腸菌を用いて発現・精製を試み、その発現と精製の可能性を見出した。

A. 研究目的

現行のHBV感染症に使用されている抗HBV剤は抗HIV剤の流用であり、HBVの本質を理解した真の抗HBV剤は開発されていない。HBVポリメラーゼ（HBVpol）の特性に基づいた、或は立体構造に根ざした迅速な抗HBV開発システムの構築を目指し、大腸菌、酵母、昆虫細胞等を利用したHBVpolの大量発現・精製を試み、活性測定系の樹立や立体構造解析によるhigh-throughput活性阻害化合物スクリーニング系の開発を試みる。今回、HBVpolの各ドメインの内、TP及びRTのGSTタグ付加発現系を大腸菌を用いて構築し発現・精製を試みた。

B. 研究方法

発現プラスミド

1) HBVpol　TPドメイン177アミノ酸をGST融合させた大腸菌発現ベクターGST-TPを構築した。
2) また同様にHBVpol　RTドメイン344アミノ酸を発現するGST-RTを構築した。
3) 上記発現プラスミドを用いてRossetta-Gami2を形質転換し、0.5mM　IPTG、30°Cで発現誘導し、抽出液を作製の上、グルタチオン-セファロースカラムを用いて精製を試みた。

（倫理面への配慮）

遺伝子組換え実験指針に従い遂行した。

C. 研究結果

1)GST-TP、GST-RTともに発現誘導が可能であった。

2) グルタチオン-セファロースを用いた精製系では、部分精製が可能であった。

D. 考察

GST-TP、GST-RT の大腸菌における発現誘導は可能であったが、大量発現に向けて精製度を高める必要があると思われた。

E. 結論

GST-TP、GST-RT の大腸菌における発現誘導は可能であり、精製度を高めることにより、特異抗体の作製、活性測定系の構築、立体構造の解明が可能であると思われた。

F. 研究発表

論文発表

無し

G. 知的所有権の出願・取得状況

1. 特許取得

該当無し

2. 実用新案登録

該当無し

III. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
上田啓次（上田）	HHV-8	新居志郎	病原細菌・ウイルス図鑑	北海道大学出版会			編集中

雑誌

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Miyoshi, E., et al. (三善)	Physiological roles of N-acetylglucosaminyltransferase V (GnT-V) in mice	BMB report (review)	45 (10)	554-559	2012
Nawa, T., et al. (竹原)	Interferon-α suppresses hepatitis B virus enhancer II activity via the protein kinase C pathway.	J.Virol.	432	452-459	2012
Shigekawa M., et al. (竹原)	Pancreatitis STAT3 protects mice against caerulein-induced pancreatitis via PAP1 induction.	Am J Pathol	181	2105-2113	2012

Kohga, K., et al. (竹原)	Interleukin-1ß enhances the production of soluble MICA in human hepatocellular carcinoma.	Cancer Immunol Immunother	61	1425-1432	2012
Hikita H., et al. (竹原)	Bak deficiency inhibits liver carcinogenesis: A causal link between apoptosis and carcinogenesis	J Hepatol	57	92-100	2012
Hosui A., et al. (竹原)	Suppression of signal transducers and activators of transcription 1 (STAT1) in tumor indicates poor prognosis	Int J Cancer	131	2774-2784	2012
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IV. 研究成果の刊行物・別刷

frontiers in
MICROBIOLOGY

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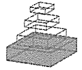

Kaposi's sarcoma-associated herpesvirus genome replication, partitioning, and maintenance in latency

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Kaposi's sarcoma-associated herpesvirus (KSHV) is thought to be an oncogenic member of the γ-herpesvirus subfamily. The virus usually establishes latency upon infection as a default infection pattern. The viral genome replicates according to the host cell cycle by recruiting the host cellular replication machinery. Among the latently expressing viral factors, LANA plays pivotal roles in viral genome replication, partitioning, and maintenance. LANA binds with two LANA-binding sites (LBS1/2) within a terminal repeat (TR) sequence and is indispensable for viral genome replication in latency. The nuclear matrix region seems to be important as a replication site, since LANA as well as cellular replication factors accumulate there and recruit the viral replication origin in latency (ori-P) by its binding activity to LBS. KSHV ori-P consists of LBS followed by a 32-bp GC-rich segment (32GC). Although it has been reported that LANA recruits cellular pre-replication complexes (pre-RC) such as origin recognition complexes (ORCs) to the ori-P through its interaction with ORCs, this mechanism does not account completely for the requirement of the 32GC. On the other hand, there are few reports about the partitioning and maintenance of the viral genome. LANA interacts with many kinds of chromosomal proteins, including Brd2/RING3, core histones, such as H2A/H2B and histone H1, and so on. The detailed molecular mechanisms by which LANA enables KSHV genome partitioning and maintenance still remain obscure. By integrating the findings reported thus far on KSHV genome replication, partitioning, and maintenance in latency, we will summarize what we know now, discuss what questions remain to be answered, and determine what needs to be done next to understand the mechanisms underlying viral replication, partitioning, and maintenance strategy.

Keywords: Kaposi's sarcoma-associated herpesvirus, human herpesvirus 8, latency-associated nuclear antigen, ori-P, DNA replication, genome maintenance, pre-replication complex, nuclear matrix

INTRODUCTION

Kaposi's sarcoma (KS)-associated herpesvirus (KSHV) is a gamma-2 herpesvirus discovered from KS specimens in 1994 (Chang et al., 1994). KSHV is closely associated with KS and several non-Hodgkin lymphomas, including primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD; Cesarman et al., 1995, 1996; Soulier et al., 1995). While KS is the most common cancer in acquired immune deficiency syndrome patients (Potthoff et al., 2010), KSHV is detected in about 95% of all types of KS lesions by PCR analysis (Dupin et al., 1995; Huang et al., 1995; Moore and Chang, 1995). PEL is a rare B cell lymphoma originated from preterminal B cells, and PEL in AIDS patients is often associated with KSHV as well as EBV. Several KSHV-infected PEL cell lines have been established, and EBV is frequently lost in the course of establishment (Arvanitakis et al., 1996; Gaidano et al., 1996; Renne et al., 1996; Said et al., 1996; Carbone et al., 1997, 1998; Katano et al., 1999). MCD is a plasmacytic lymphadenopathy with polyclonal hyper-immunoglobulinemia and high levels of serum IL-6 (Frizzera et al., 1983; Yoshizaki et al., 1989).

Like all herpesviruses, KSHV has two life cycles: latent and lytic replication phases (for review, see Boshoff and Chang, 2001). Whereas KSHV is usually in latency when it infects KS and PEL cells, in MCD some cells express lytic genes (Katano et al., 2000; Parravicini et al., 2000). On the other hand, it has been reported that KSHV infection itself and/or viral lytic proteins promote cell proliferation and angiogenesis as well as lymphatic reprogramming (Ciufo et al., 2001; Gao et al., 2003; Carroll et al., 2004; Hong et al., 2004; Naranatt et al., 2004; Pan et al., 2004; Wang et al., 2004; Sharma-Walia et al., 2006; Qian et al., 2007, 2008; Sadagopan et al., 2007; Ye et al., 2007).

In latency, the KSHV genome is present as an episome, which is capable of autonomously replicating during S phase of the host cell cycle without integration into host chromosomes, and only limited genes are expressed during latency. Therefore, there is no generation of progeny virions. It is very important to elucidate and learn the virus's survival strategy in order to control infection and to formulate treatment for KSHV-related diseases.

In this review, we would like to focus on studies on the mechanisms underlying viral DNA replication, genome segregation and maintenance, and gene expression regulation in latency, and to discuss these topics in the light of studies on cellular mechanisms.

GENE EXPRESSION CONTROL IN KSHV LATENCY

The KSHV genome is a double-stranded linear DNA in the virion. It is circularized upon infection and is maintained as an episome in the infected nucleus. The complete genome is about 160–170 kbp,

including a 40 ∼ 50 times repeated sequence called a terminal repeat (TR), which is 801 bp as a unit, at each end of the genome (**Figure 1**). The viral genome encodes approximately 90 ORFs in the unique region (for reviews, see Moore and Chang, 2001; Dourmishev et al., 2003). In latency, the viral genome is maintained in a cell cycle-dependent manner, and extremely limited viral genes, such as latent gene clusters, are expressed. Although the establishment of latent infection could be this virus's default infection mode, it is not good for the virus to continue latent infection for a long time, since it will vanish from the earth without progeny virus production. Thus, latency is a state in which the virus waits for an opportunity for the explosive production of progeny virions. Nevertheless, it is very interesting how KSHV establishes latency and is maintained in infected host cells without losing the genomes.

As mentioned above, the limited region within the KSHV genome is transcriptionally active in latency, and this region contains only several genes, including the latency-associated nuclear antigen (Wang and Frappier, 2009), viral cyclin (*v-CYC*), viral FLICE-inhibitory protein (*v-FLIP*), *kaposin*, 17 microRNAs (miRNAs), and viral interferon regulatory factor 3 (*v-IRF-3*; Chang et al., 1996; Thome et al., 1997; Muralidhar et al., 1998; Lubyova and Pitha, 2000; Gomez-Roman et al., 2001; Rivas et al., 2001; Staudt and Dittmer, 2003; Cai et al., 2005; Pearce et al., 2005; Pfeffer et al., 2005; Samols et al., 2005). Such genes, except for *v-IRF-3*, are in that limited region, and *LANA*, v-*CYC*, and *v-FLIP* are in one of the unit's genes. This region forms an active locus for expression including miRNAs and *kaposin*. It is unclear why this region is active for the expression of genes and is insulated from inactive lytic genes, such as ORF69 and K14 just downstream and upstream, respectively.

Recently, it was reported that CTCF, which is the only insulator protein found in vertebrates, coupled with SMC3, endows this gene insulation. More interestingly, the binding sites are in the genes, i.e., downstream from the *LANA* transcription start sites, not at the boundary regions (Stedman et al., 2008; Kang and Lieberman, 2009). Transcriptional analysis using the KSHV-BAC system demonstrated that mutations of CTCF binding sites abolished latency-regulated transcription such as K14 and ORF74 during latency (Kang and Lieberman, 2009). CTCF usually binds at the boundary regions between active and inactive loci in mammalian genomes, forming locus control regions (LCRs; Tanimoto et al., 2003). A typical example is an LCR seen in the beta-globin locus. CTCF binds to several DNase I hypersensitivity sites (HS), called HS4 and HS5, and forms boundaries to insulate this locus from the outside locus (Tanimoto et al., 2003; Hou et al., 2008). Thus, latent gene expression in KSHV-infected cells might be regulated differently from the mechanism observed in the beta-globin locus.

Inversely, it is interesting how the viral lytic genes are tightly inactivated in latency. Epigenetic regulation seems to be essential for inactivation as well as activation of latent genes. LANA recruits heterochromatin components to the TR by the interaction between LANA and SUV39H1, which is a key factor that methylates histone H3, which in turn recruits heterochromatin protein 1 (HP1; Sakakibara et al., 2004). Because this mechanism contributes to the propagation and maintenance of heterochromatin, it appears that heterochromatin could spread over the KSHV genome during latency. The propagation of heterochromatin into the active latent gene zone might be blocked by the boundary effect and by the enhancer-blocking activity of an insulator, CTCF which has multiple functions such as gene activation or inactivation, X-chromosome inactivation, and gene imprinting (for review, see Zlatanova and Caiafa, 2009).

Thus, it is thought that not the overall lytic genes region, except for the latent gene clusters, forms heterochromatin during latency, because recent genome-wide analysis using ChIP-on-chip showed that not only latent gene clusters but also several regions of lytic genes are enriched in activating histone marks (acetylated H3 and H3K4me3). However, H3K27me3, which is a bivalent histone marker, is widely distributed through the KSHV genome (Toth et al., 2010), meaning that the genome is poised for reactivation. Furthermore, the treatment of specific histone demethylases of H3K27me3 such as JMJD3 and UTX could induce the lytic reactivation. Immunoprecipitation of methylated DNA assay showed that the KSHV genome was methylated during latency (Gunther and Grundhoff, 2010). Gunther and Grundhoff (2010) suggested that the CpG methylation process could take a long time to prevail over the genome, and thus could not control early latency. There are several reports that DNA methylation of viral genomes is related to the regulation of the gene expression of gammaherpesviruses such as EBV and herpesvirus saimiri (HVS; Minarovits, 2006). Heterochromatin formation on the viral genomes, however, seems to be inconvenient for the rapid induction of lytic replication. Further investigations are needed to clarify how viruses are ready for lytic induction if heterochromatin and/or DNA methylation was formed on the genome.

Viral factors play key roles in maintaining gene expression profiles in latency. Otherwise, modulation by viral and cellular factors maintains viral latency. In addition to the recruitment of

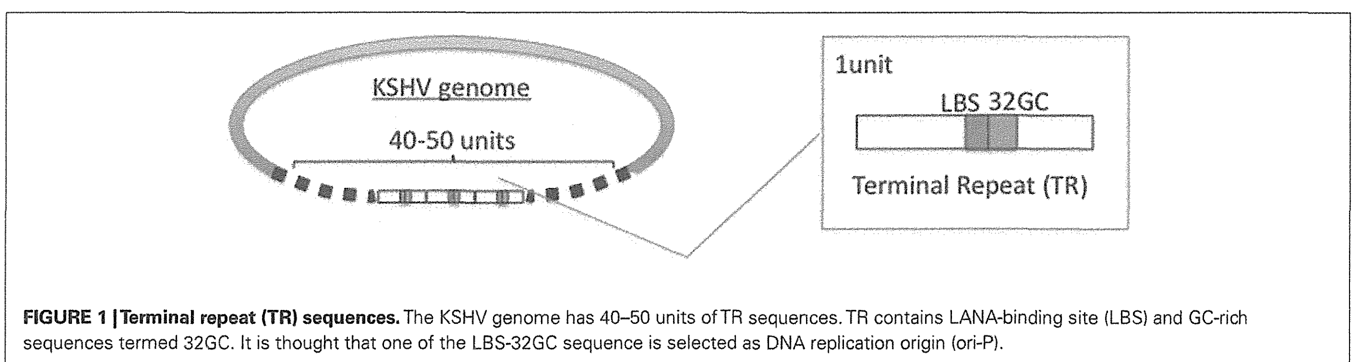

FIGURE 1 | Terminal repeat (TR) sequences. The KSHV genome has 40–50 units of TR sequences. TR contains LANA-binding site (LBS) and GC-rich sequences termed 32GC. It is thought that one of the LBS-32GC sequence is selected as DNA replication origin (ori-P).

heterochromatin factors on to the genome, LANA itself tends to repress viral lytic gene expression. LANA physically associates with recombination signal sequence-binding protein Jκ (RBP-Jκ) and represses the replication and transcription activator (RTA) promoter through the RBP-Jκ binding site existing within its promoter (Lan et al., 2005a).

Viral FLICE-inhibitory protein, also known as K13, interacts with several NF-κB-related signaling proteins and activates the NF-κB pathways, thus enhancing cell survival (Chaudhary et al., 1999; Field et al., 2003; Matta et al., 2003, 2007; Matta and Chaudhary, 2004). It should be noted that the effect of NF-κB signaling on reactivation depends on cellular context (Grossmann and Ganem, 2008) and seems to be regulated by an intricate balance within the cellular environment. Previous studies, however, demonstrated that v-FLIP repressed the RTA promoter by activating NF-κB binding to the cognate sequence, but in that case it deregulates vIL-6 and hIL-6 expression (Zhao et al., 2007). The reactivation is initiated by RTA, which is a lytic switch protein and a homolog of EBV BRLF1 (Liang et al., 2002). The RTA promoter region is highly responsive to 12-*O*-tetradecanoylphorbol 13-acetate (TPA) or phorbol 12-myristate 13-acetate (PMA), sodium butylate (NaB), and trichostatin A (TSA), and is associated with several histone deacetylase proteins such as HDAC, which leads to chromatin remodeling of a nucleosome and then regulates KSHV reactivation from latency (Lu et al., 2003). RTA activates various viral genes through direct binding with RTA-responsive elements existing within the K8 and ORF57 promoters (Byun et al., 2002) and also through indirect mechanisms on RTA itself and vIRF-1 (Nishimura et al., 2001; Sakakibara et al., 2001; Ueda et al., 2002). Although RTA is a strong transactivator and inducer of lytic replication, it also enhances LANA expression and then is involved in the establishment of latency in the early infection phase (Lan et al., 2005b). This feedback mechanism explains the low efficiency of lytic replication and the generation of complete viral particles in KSHV-infected cell lines.

MicroRNAs (miRNAs) are single-stranded and 20- to 23-nucleotide RNA molecules that are involved in gene expression (Bartel, 2004; Bartel and Chen, 2004). Recent studies have highlighted the critical role of viral microRNAs (miRNAs) in the maintenance of KSHV latency (for review, see Ganem and Ziegelbauer, 2008; Boss et al., 2009; Lei et al., 2010a). The KSHV genome contains 17 miRNAs that are clustered and located in the intragenic region between *kaposin* and *v-FLIP* (Cai et al., 2005; Pearce et al., 2005; Samols et al., 2005; Cai and Cullen, 2006). Surprisingly, a new proteomic approach suggests that a single miRNA can directly lead to the suppression of the synthesis of hundreds of proteins at both mRNA and translation levels, although the level of suppression is mild (Baek et al., 2008; Selbach et al., 2008).

Kaposi's sarcoma-associated herpesvirus miRNAs are reported to regulate, directly or indirectly, various factors including lytic genes (Murphy et al., 2008; Bellare and Ganem, 2009) and cellular factors such as NF-κB and IκBα, the latter of which is directly regulated by KSHV miR-K1 (Lei et al., 2010b) and Bcl-2 associated factor (*BCLAF1*) as a target of miR-K5 (Ziegelbauer et al., 2009). miR-K12-7 (Lin et al., 2011) and miR-K9 (Bellare and Ganem, 2009) directly target RTA and contribute to the maintenance of latency. miR-K12-11 shows remarkable homology to cellular miR-155;it inhibits a BACH-1 3′UTR-containing reporter and downregulates the expression of BACH-1, which is a broadly expressed transcriptional repressor that regulates genes involved in the hypoxia response (Gottwein et al., 2007; Skalsky et al., 2007). Thrombospondin 1 (*THBS1*), an inhibitor of angiogenesis, is targeted by multiple KSHV miRNAs, such as miR-K12-1, miR-K12-3-3p, miR-K12-6-3p, and miR-K12-11(Samols et al., 2007). miR-K1 represses the expression of p21 via the 3′UTR and attenuates p21-mediated cell cycle arrest during KSHV latency (Gottwein and Cullen, 2010).

CELLULAR DNA LICENSING AND VIRAL DNA REPLICATION IN LATENCY

Because eukaryotic DNA replication is strictly regulated by a licensing mechanism, the genome is replicated only once per cell cycle. DNA replication starts at multiple sites on a chromosome; these sites are called the replication origin, whose number is predicted to be 30,000 ~ 50,000 (Huberman and Riggs, 1966). The genome size of eukaryotes is about 10^7 to 10^{11} bp (fungi to mammals), and the entire DNA must be replicated within a limited time (Wyrick et al., 2001). To achieve this, many proteins participate in DNA replication licensing, including origin recognition complex (ORC), Cdc6, Cdt1, and mini-chromosomal maintenance (MCM) helicase, and so on. First, ORC recognizes and binds to the origins and then recruits a Cdc6 followed by the association of another replication protein, called Cdt1. Finally, the MCM helicase is loaded onto the complex to establish a complete pre-replication complex (pre-RC) (reviews in Nishitani and Lygerou, 2002; DePamphilis, 2003, 2005).

KSHV ORIGIN OF REPLICATION IN LATENCY

The features of DNA replication origins have been reported. Although there are no consensus sequences for the replication origin, recent studies showed that CpG islands, promoter regions, DNA topology, and nucleosome positioning are involved in origin selection (Mechali, 2010). *Saccharomyces cerevisiae* (*S. cerevisiae*) has autonomous replication sequence (ARS) elements that are specific 12 bp consensus sequences and has origin activity (Stinchcomb et al., 1979; Bell and Stillman, 1992). *S. pombe* ARS also has been identified, but it does not share a consensus sequences as in *S. cerevisiae* (Segurado et al., 2003; Dai et al., 2005; Heichinger et al., 2006). Substitution experiments showed that the ARS region could be replaced with a 40-bp poly (dA/dT) fragment (Okuno et al., 1999).

In higher eukaryotes, no consensus sequences are identified, though known origin sequences have been reported (for review, see Aladjem, 2004). It is not yet known why there is no consensus sequence among ORC binding sites of higher eukaryotes, or how they are selected. Replication origins should be determined by different mechanisms, and recent genome-wide analyses show that the origin sequences are closely related with transcriptional regulatory elements and CpG islands but not sequence motifs (Cadoret et al., 2008; Sequeira-Mendes et al., 2009).

The KSHV genome appears to replicate once per cell cycle during latency, as cellular DNA replication. The number of the genome copies is supposed to be 50–100 per KSHV-infected PEL cell and the copy number is kept at the same number, at least

appears to be kept at the same copy number (Cesarman et al., 1995; Ballestas et al., 1999; Ueda et al., 2006). This observation suggests that the KSHV genome uses cellular replication machinery so that viral DNA replication synchronizes with the cell cycle. The KSHV origin of replication in latency, called ori-P, consists of two LANA-binding sites (LBS), in which LBS1 has a higher affinity with LANA (Garber et al., 2002), and the following 32 bp GC-rich segment (termed 32GC in this manuscript) and the ori-P is in the TR region of the KSHV episome (Hu and Renne, 2005; **Figure 1**). LANA directly binds to LBS and supports viral DNA replication (Garber et al., 2002; Hu et al., 2002; Fejer et al., 2003; Grundhoff and Ganem, 2003). The components of pre-RC, such as ORC, Cdc6, and MCM were recruited to the TR sequences in a LANA-dependent manner (Lim et al., 2002; Ohsaki et al., 2004; Stedman et al., 2004; Verma et al., 2006).

In the case of EBV ori-P, the dyad symmetry (DS) and family of repeats (FR) are essential for the ori-P activity (Reisman et al., 1985), though FR rather works for viral genome maintenance. The DS element contains two EBNA1 binding sites and is the functional replicator in the presence of EBNA1 (Wysokenski and Yates, 1989; Harrison et al., 1994). The FR element contains 20 copies of a 30-bp repeat sequence and has an essential role in the long-term maintenance of ori-P-containing plasmid (Krysan et al., 1989; Marechal et al., 1999). It is suggested that cellular replication factors bind to the sequences adjacent to EBNA1 binding sites through the interaction with EBNA1 (Yates et al., 2000; Koons et al., 2001). A chromatin immunoprecipitation assay suggested that the ORC complex and EBNA1 bound to chromatin and ori-P in G0-arrested cells (Ritzi et al., 2003). Nucleosome assembly proteins, such as NAP1 and TAF-I, interact with EBNA1 and are recruited to the ori-P regions. These proteins contribute to the activation of transcription, although TAF-I negatively regulates DNA replication (Wang and Frappier, 2009). The EBNA1 LR1 and LR2 domains are critical for the interaction with ORC and for disrupting this association by binding with G-rich RNA (Norseen et al., 2009). Thus, the latent replication of KSHV and EBV totally depends on cellular DNA replication machinery with the only exception of the requirement of LANA and EBNA1, respectively.

HOW LANA WORKS

LANA is a nuclear protein with 1162 amino acids. It shows a functional homology to EBNA1 of EBV and, in part, to E1/E2 of human papillomavirus and to SV40 large T antigen. Especially, the C-terminus of LANA and that of EBNA1 conserve secondary and tertiary structures (Han et al., 2010). The N-terminus of LANA contains a chromosome binding site (CBS) and a nuclear localization signal (NLS), and the C-terminus contains a DNA binding domain and a dimerization domain, called DBD. In the DBD, there appears to be another NLS, but this NLS is rather cryptic and weak, since an N-terminal deleted mutant, which contains 108–1162 aa, is localized in the cytoplasm (Ohsaki et al., 2009). The central region is composed of a proline-rich region, an aspartate (D)- and glutamate (E)-rich repetitious region, and a glutamine-rich domain (Garber et al., 2001; Piolot et al., 2001; **Figure 2**). DBD (923–1162 aa) is necessary and partially sufficient to support ori-P replication compared to the full-length LANA (Hu et al., 2002; Ohsaki et al., 2009), and when considering their expression levels in *in vitro* study.

Many studies show that LANA binds to LBS and recruits ORC to the origin as described above. What is the mechanism by which ORC is recruited to ori-P? One possible mechanism is that LANA directly interacts with ORC so that ORC is loaded to the origin (Lim et al., 2002; Stedman et al., 2004; Verma et al., 2006). If LANA could directly interact with ORC and recruit to the origin, we are confronted with this question: why is 32GC required for ori-P activity despite LANA's ability to bind to the ori-P? It could be that the 32GC is required to load ORC and the other pre-RC components to the region next to LBS, although the underlying molecular mechanism is not yet known. Further study is needed to resolve this question.

LANA is highly expressed in KSHV-related malignancies, and plays an essential in episomal maintenance. It interacts with multiple cellular proteins, including tumor suppressors such as p53 (Friborg et al., 1999) and Rb (Radkov et al., 2000), as well as transcription factors such as ATF4/CREB2 (Lim et al., 2000) and STAT3 (Muromoto et al., 2006), chromatin-associated proteins such as HP1 (Lim et al., 2003), histone H2A/B (Barbera et al., 2006b), MeCP2 (Krithivas et al., 2002; Matsumura et al., 2010), and Brd4 (Ottinger et al., 2006), in addition to signal transducers such as GSK-3b (Fujimuro and Hayward, 2003) and so on (**Figure 2**). Almost all of these proteins interact with the C-terminal domain of LANA, implying the functional importance of this domain. However, careful analysis of protein–protein interaction is required, because only DBD sometimes shows non-specific binding with other proteins and may exhibit different properties from the full-length LANA (our personal observation).

REGULATION OF KSHV ori-P ACTIVATION

The origin number varies from species to species. In mammals, it, 30,000–50,000 origins are thought to exist at each cell cycle (Huberman and Riggs, 1966). However, not all of these origins start DNA synthesis at the same time. Some origins are activated early in the S phase, whereas others are activated in the late S phase; that is, the DNA replication timing is controlled (Dimitrova and Gilbert, 1999; Cimbora et al., 2000). How is the timing of DNA replication determined? Some groups have provided possible answers to this question. It was reported that heterochromatin could change the timing of DNA replication by transgene insertion into a mammalian genome (Lin et al., 2003). Sir proteins, which are silencer proteins, can delay replication and correlate with transcriptional silencing (Zappulla et al., 2002). Thus, various studies suggest that heterochromatin modulates replication timing (Goren et al., 2008; Klochkov et al., 2009; Schwaiger et al., 2010) and EBV replication in latency occurs in mid-late S phase (Zhou et al., 2009). In the case of KSHV, the replication timing of the viral genome is not yet known. Considering that ori-P is present in the proximity of heterochromatin because of LANA-dependent accumulation of heterochromatin, DNA replication of the KSHV genome may start at the middle or late S phase.

The frequency of origin usage also differs from origin to origin; some origins are used in every cell cycle, whereas others are used rarely. Various studies including in *Drosophila*, *Xenopus*, and mammals demonstrate how specific origins are selected, but several

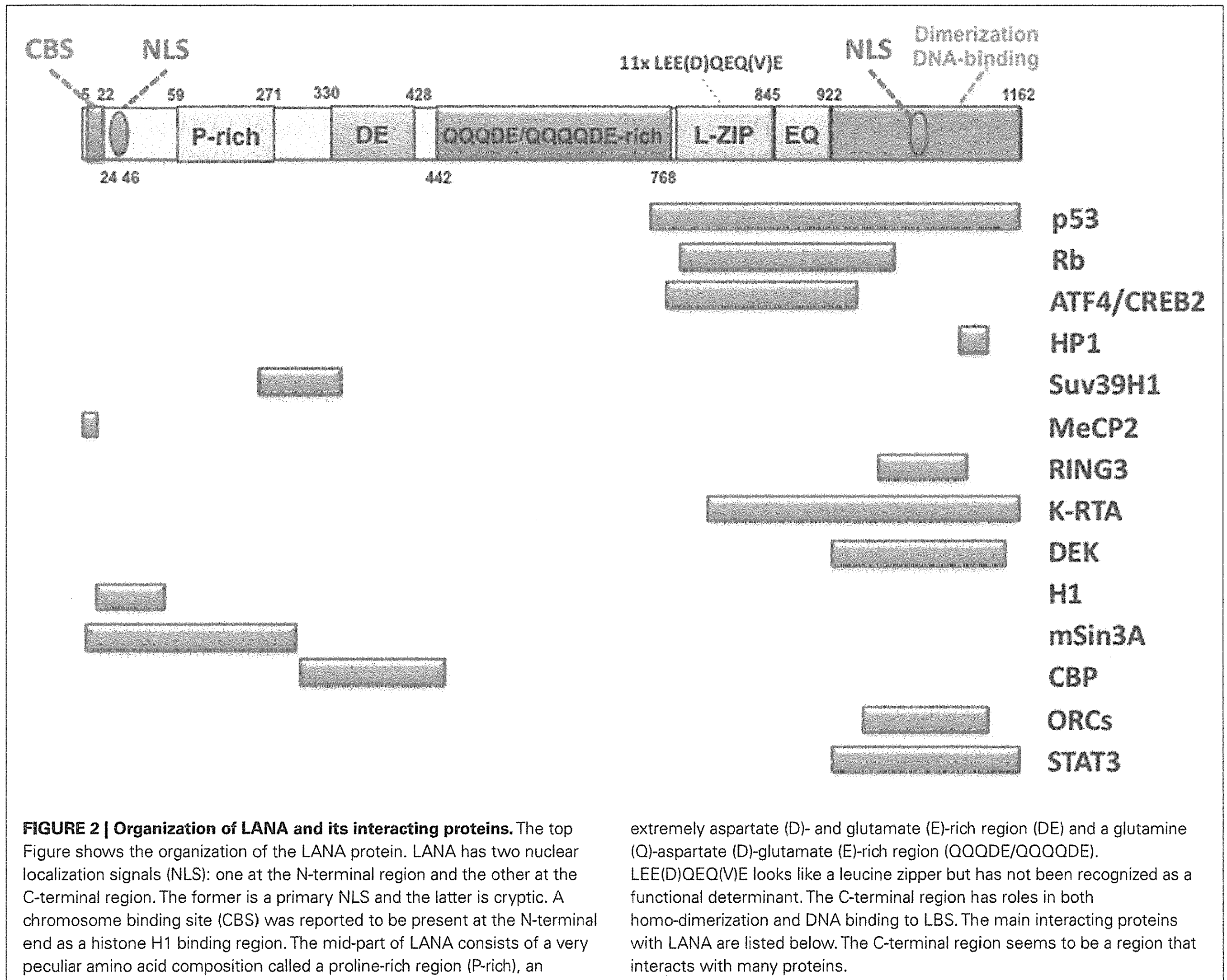

FIGURE 2 | Organization of LANA and its interacting proteins. The top Figure shows the organization of the LANA protein. LANA has two nuclear localization signals (NLS): one at the N-terminal region and the other at the C-terminal region. The former is a primary NLS and the latter is cryptic. A chromosome binding site (CBS) was reported to be present at the N-terminal end as a histone H1 binding region. The mid-part of LANA consists of a very peculiar amino acid composition called a proline-rich region (P-rich), an extremely aspartate (D)- and glutamate (E)-rich region (DE) and a glutamine (Q)-aspartate (D)-glutamate (E)-rich region (QQQDE/QQQQDE). LEE(D)QEQ(V)E looks like a leucine zipper but has not been recognized as a functional determinant. The C-terminal region has roles in both homo-dimerization and DNA binding to LBS. The main interacting proteins with LANA are listed below. The C-terminal region seems to be a region that interacts with many proteins.

questions about the decision mechanism of origins remain unanswered. Considering the genome size of this virus, one origin is enough to complete replication within the S phase; and because of the existence of "origin interference" by the ATR and ATM pathways (Shechter et al., 2004), a single origin must be chosen.

The micrococcal nuclease digestion pattern at TR in G1-arrested cells leads to change, suggesting that the chromatin structure became more accessible to enzymatic digestion (Stedman et al., 2004). Therefore, the chromatin structure may be changed by the recruitment of the replication machinery during the late G1 phase.

A recent study shows that the cellular deubiquitylating enzyme USP7 stimulates EBNA1 binding to its recognition sites so that histone modification at the EBV ori-P is changed by EBNA1 mediating the recruitment of USP7 (Sarkari et al., 2009).

THE ROLES OF NUCLEAR ARCHITECTURES

NUCLEAR MATRIX AS A SCAFFOLD FOR DNA REPLICATION AND TRANSCRIPTION

The nucleus consists of a well-organized structure and is highly complex. The structures of nuclear matrix proteins such as lamins, nuclear mitotic apparatus (NuMA), hnRNP, and so on, are important for the organization of chromatin, DNA replication, and transcription (Dechat et al., 2008). The nuclear matrix, isolated by Berezney and Coffey (1974), is believed to support the spatial distribution of several nuclear factors, such as DNA replication machinery and transcription factors. The nuclear matrix fraction contains DNase I-resistant and high salt-resistant proteins. Because the nuclear matrix can be visualized only after chromatin extraction, there has been a debate that such a nuclear matrix is an essential component of *in vivo* nuclear architectures. In previous studies, replication origins have come to the nuclear matrix (van der Velden et al., 1984; Amati and Gasser, 1990; Adom et al., 1992; Brylawski et al., 1993; Fallaux et al., 1996). Eukaryotic DNA is organized into DNA loops generated by the attachment of chromatin to the nuclear matrix via specific regions, referred to as scaffold/matrix attachment regions (Pardoll et al., 1980; Vogelstein et al., 1980; Laemmli et al., 1992; Roberge and Gasser, 1992). DNA loop formation is essential for DNA replication, transcription, and chromosomal packaging (Gasser and Laemmli, 1987; Berezney et al., 1995; Bode et al., 1995; Nickerson et al., 1995; Razin et al., 1995; Jackson, 1997; Volpi et al.,