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

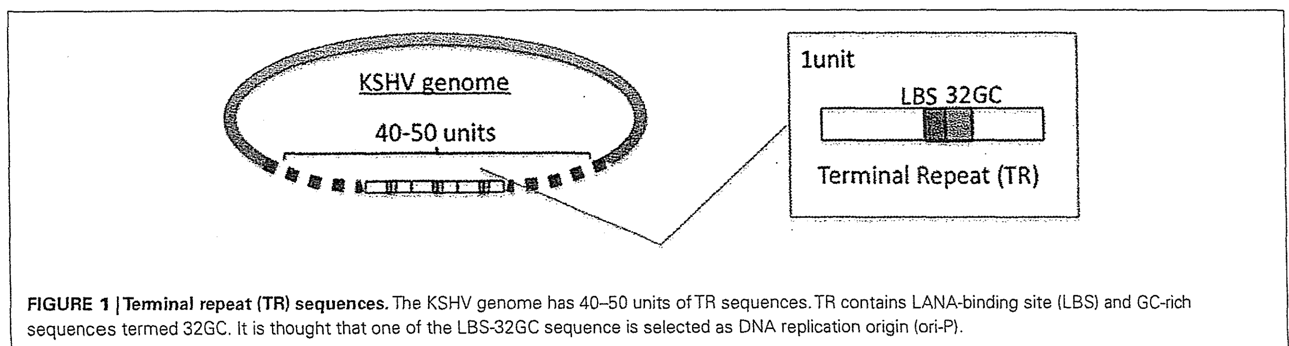

FIGURE 1 | Terminal repeat (TR) sequences. The KSHV genome has 40–50 units of TR sequences. TR contains LANA-binding site (LBS) and GC-rich sequences termed 32GC. It is thought that one of the LBS-32GC sequence is selected as DNA replication origin (ori-P).

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

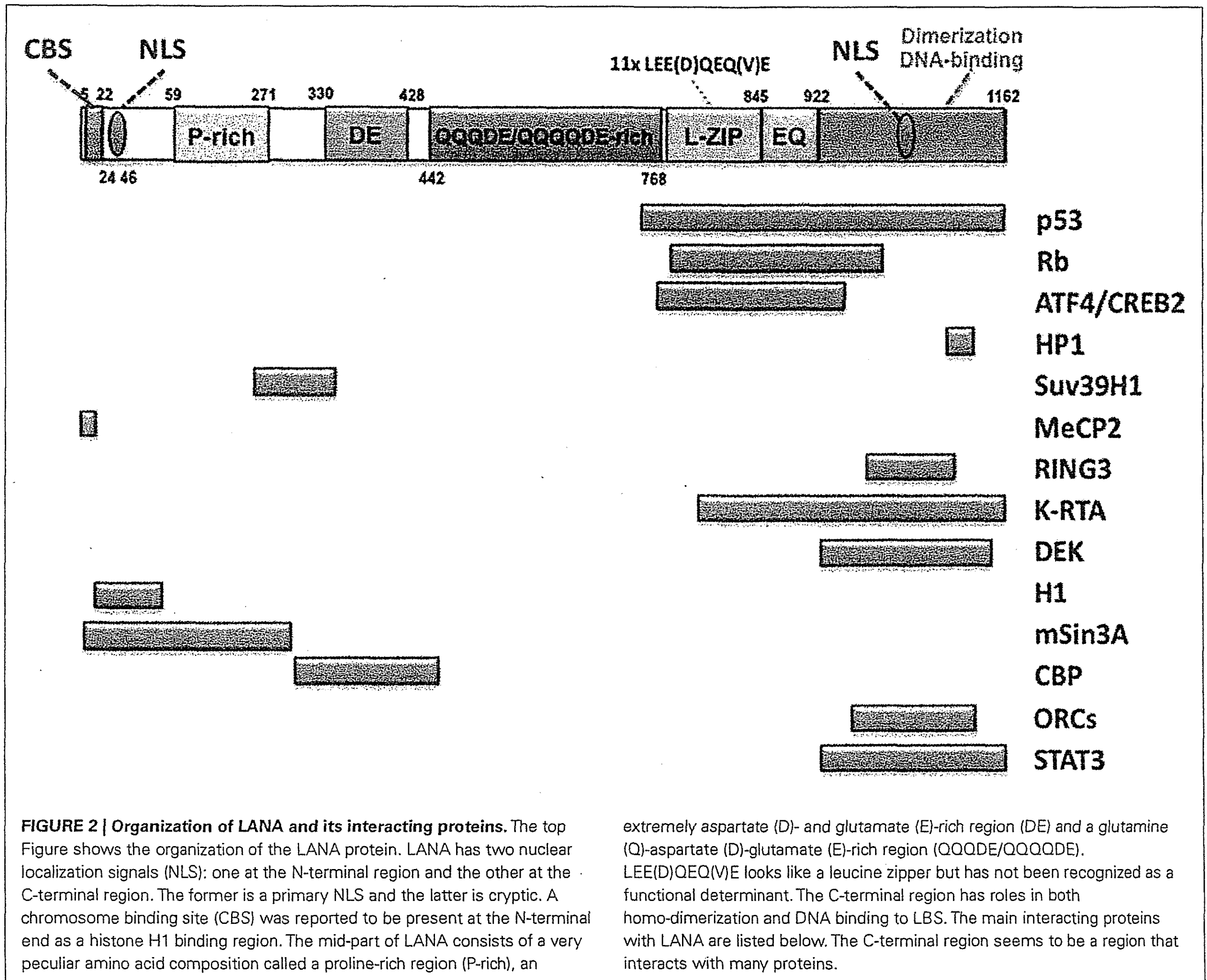

FIGURE 2 | Organization of LANA and its interacting proteins. The top Figure shows the organization of the LANA protein. LANA has two nuclear localization signals (NLS): one at the N-terminal region and the other at the C-terminal region. The former is a primary NLS and the latter is cryptic. A chromosome 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.,

2000; Mahy et al., 2002; Sumer et al., 2003; Marenduzzo et al., 2007).

In EBV, both the latent ori-P and the lytic ori-Lyt are bound to the nuclear matrix (Jankelevich et al., 1992; Mattia et al., 1999). EBV nuclear antigen leader protein (EBNA-LP) is a nuclear matrix-associated protein, and its nuclear matrix-targeting signal is a 10-aa segment, which also functions as a NLS (Yokoyama et al., 2001). However, this is not the case for LANA, because it was reported that C-terminal truncation up to 1128 aa could not be localized in a high salt-resistant fraction (nuclear matrix fraction), but the C-terminal region (1129–1143 aa) is high salt extractable (Viejo-Borbolla et al., 2003). We previously showed that the N-terminal region up to 107 aa is localized in nucleo-cytoplasmic and chromatin fractions (Ohsaki et al., 2009). The localization to the nuclear matrix fraction of LANA might depend on the conformation of LANA or post-translational modifications.

On the other hand, a cell fractionation assay has shown that cellular pre-RC components, such as ORC2, Cdc6, and Cdt1, preferentially localize in the nuclear matrix fraction in a cell cycle-dependent manner, and LANA itself also can localize in the nuclear matrix fraction. Accordingly, TR accumulates in the nuclear matrix fraction during the late G1 phase, suggesting that LANA recruits the ori-P to the nuclear matrix, so that cellular replication machinery is abundant and available for viral DNA replication during latency (Ohsaki et al., 2009; **Figure 3**).

GENOME SEGREGATION MECHANISMS OF KSHV IN LATENCY

The same copy number of KSHV genomes appears to be maintained in daughter cells after every cell division (Ballestas et al., 1999; Ueda et al., 2006), indicating that a strict genome maintenance mechanism is working. In the KSHV-infected PEL cell lines, LANA associates with a condensed mitotic chromatin (Ballestas et al., 1999; Cotter and Robertson, 1999; Tetsuka et al., 2004). It was reported that MeCP2, a methyl CpG-binding protein, interacts with the N-terminal of LANA and that DEK protein interacts with the C-terminal of LANA. These two independent interactions are involved in the tethering of LANA to chromosomes (Krithivas et al., 2002). Various studies have reported the interaction between LANA and multiple cellular proteins associated with chromatin.

Brd4, which is a member of the BET family that carries two bromodomains and associates with mitotic chromosomes, interacts with LANA on mitotic chromosomes (You et al., 2006). It has also been reported that core histones such as H2A and H2B are essential for LANA N-terminal chromosome binding (Barbera et al., 2006a,b). Furthermore, LANA interacts with Brd2/Ring3, which is a member of the BET family of double bromodomain-containing genes and contains two tandem bromodomains (Viejo-Borbolla et al., 2005).

Xiao et al. (2010) reported that LANA is associated with centromeres via the formation of complexes with Cenp-F and Bub1, which are kinetochore-associated proteins. This suggests that LANA preferentially interacts with kinetochore-associated proteins and that its association is critical for segregation into daughter cells. Though the interaction of LANA with kinetochore factors might interfere with correct spindle formation, it suggests that LANA should support viral genome segregation along with condensed chromatin.

A NuMA plays a critical role in the nuclear architecture in the interphase. After nuclear envelope breakdown in mitosis, NuMA is hyperphosphorylated by $p34^{cdc2}$ and is distributed at spindle poles, where it remains until the anaphase and plays an essential role in tethering spindle microtubules to each pole (Merdes et al., 1996; Gehmlich et al., 2004). Although NuMA drastically alters the localization and functions in the interphase and M phase, biochemical fractionation analysis shows that NuMA is localized in

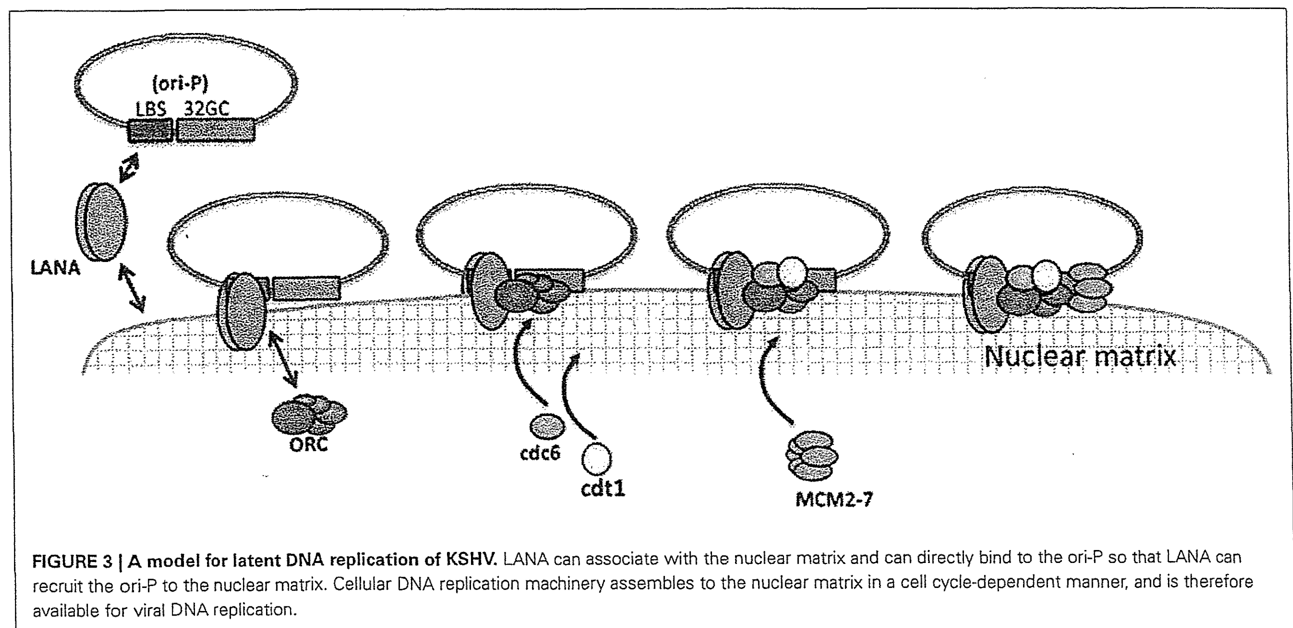

FIGURE 3 | A model for latent DNA replication of KSHV. LANA can associate with the nuclear matrix and can directly bind to the ori-P so that LANA can recruit the ori-P to the nuclear matrix. Cellular DNA replication machinery assembles to the nuclear matrix in a cell cycle-dependent manner, and is therefore available for viral DNA replication.

the insoluble nuclear matrix fraction in both phases. A recent study demonstrated that the C-terminus of LANA was co-localized with NuMA during the interphase and that the knockdown of NuMA expression caused the disruption of genome segregation and TR-containing plasmid maintenance (Bhaumik et al., 2008) and thus, KSHV genome segregation is disrupted in the absence of NuMA. Through siRNA and knockdown strategies in mice, NuMA has been shown to be an essential protein for early embryogenesis and cellular proliferation (Harborth et al., 2001; Silk et al., 2009), and it is thus unclear how the interaction with LANA actually works for viral genome segregation.

In either case, LANA has the capability of associating with various cellular proteins so that KSHV can maintain the genome stably if the cells are divided into two daughter cells. Nuclear matrix proteins function as a scaffold of DNA replication, transcription, and repair during the interphase, and also play an essential role in the segregation of condensed chromosomes in mitosis. Condensed chromosomes include, for example, NuMA, which can behave as a component of a spindle pole during mitosis, or Cenp-F, which is a nuclear matrix protein during the interphase and is distributed to kinetochores in mitosis. Taken together, the previous and present results suggest that nuclear architectures such as the nuclear matrix have essential roles not only in DNA replication and transcription but also in genome segregation during mitosis (**Figure 4**).

CONCLUSION AND PERSPECTIVES FOR FUTURE ANALYSIS

The importance of nuclear architecture is increasingly recognized as important in various nuclear events, such as DNA replication, transcription, and DNA repair. It is well accepted that the chromosomes are organized into distinct territories in the interphase. These distributions of chromosomes are closely related to the place for active or inactive transcription, the presence of DNA replication machinery, and the formation of higher-order structures of chromatin loops. DNA looping appears to be mediated by attachment to the nuclear matrix and thus achieves transcriptional control (Ostermeier et al., 2003). Other studies have suggested that the gene-rich chromosomes are frequently located in the nuclear interior. On the other hand, gene-poor chromosomes are located in the nuclear periphery (Tanabe et al., 2002; Reddy et al., 2008). A recent study shows that the transcriptional silencing might be accomplished by the binding of a specific promoter region to lamin type A (Lee et al., 2009). It is reported that replication foci at the middle to late S phase are also preferentially located toward the nuclear periphery, whereas early replication foci are located in the nuclear interior (Grasser et al., 2008) or throughout the nucleus (Izumi et al., 2004).

The spatial and temporal analyses using live-cell imaging revealed that replication forks are generated at the same origin and are closely associated during replication (Kitamura et al., 2006). Interestingly, two replication loci, located at the same distance from the origin, were in closer proximity when DNA replication took place at these loci, after which they moved apart from each other after replication. It is speculated that the replication factory anchors some region and that replicated DNA can move away from a replication factory immediately after DNA synthesis. This anchored region may be a nuclear scaffold such as the nuclear matrix.

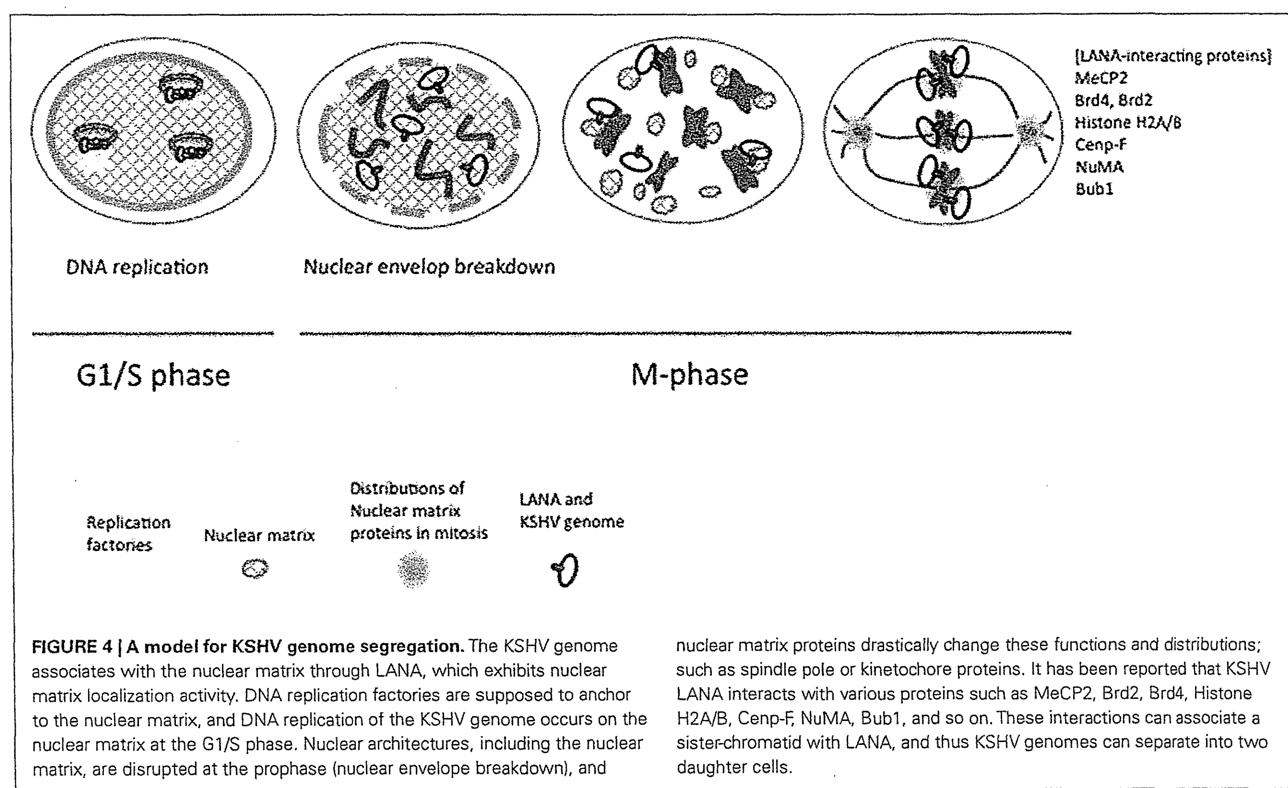

FIGURE 4 | A model for KSHV genome segregation. The KSHV genome associates with the nuclear matrix through LANA, which exhibits nuclear matrix localization activity. DNA replication factories are supposed to anchor to the nuclear matrix, and DNA replication of the KSHV genome occurs on the nuclear matrix at the G1/S phase. Nuclear architectures, including the nuclear matrix, are disrupted at the prophase (nuclear envelope breakdown), and nuclear matrix proteins drastically change these functions and distributions; such as spindle pole or kinetochore proteins. It has been reported that KSHV LANA interacts with various proteins such as MeCP2, Brd2, Brd4, Histone H2A/B, Cenp-F, NuMA, Bub1, and so on. These interactions can associate a sister-chromatid with LANA, and thus KSHV genomes can separate into two daughter cells.

The cellular environment is critical for viral survival, for which viruses make full use of the cellular machinery. The analysis of insoluble proteins, including nuclear matrix proteins, is difficult for the investigation of protein–protein and protein–DNA interactions *in vitro*, because the conditions of *in vitro* experiments always include soluble fractions. To overcome these problems, recent new approaches, such as genome-wide analysis using chromatin immunoprecipitation or live-cell imaging that reflects the *in vivo* environment, may be more powerful and accurate.

As for KSHV genome replication and maintenance, it would be first necessary to clarify how LANA is involved in both. Considering the real and actual cellular environments, we need to develop more powerful tools to know what the virus does in cells.

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Novel monoclonal antibodies for identification of multicentric Castleman's disease; Kaposi's sarcoma-associated herpesvirus-encoded vMIP-I and vMIP-II

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ABSTRACT

Recent studies have indicated that vMIP-I and vMIP-II play important roles in the pathogenesis of Kaposi's sarcoma-associated herpesvirus (KSHV)-related diseases due to the effects of these proteins on vascularization. We developed monoclonal antibodies against KSHV-encoded viral macrophage inflammatory protein-I (vMIP-I) and vMIP-II to study these expression profiles and reveal the pathogenesis of KSHV-related diseases. The MAbs against vMIP-I and vMIP-II reacted to KSHV-infected cell lines after lytic induction. Both vMIP-I and the vMIP-II gene products were detected 24 h post-induction with 12-O-tetradecanoylphorbol-13-acetate until 60 h in the cytoplasm of primary effusion lymphoma cell lines. In clinical specimens, both vMIP-I and vMIP-II gene products were detected in the tissues of patients with multicentric Castleman's disease. On the other hand, only vMIP-II was detected in a subset of Kaposi's sarcoma. We concluded that these antibodies might be powerful tools to elucidate the pathogenesis of KSHV-related diseases.

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Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), is a gammaherpesvirus originally identified in HIV-positive Kaposi's sarcoma (KS) tissues (Chang et al., 1994). KSHV is responsible for AIDS associated cancers such as Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and multicentric Castleman's disease (MCD) (Cesarman et al., 1995; Schalling et al., 1995; Soulier et al., 1995). As is the case for all herpesviruses, KSHV has two life cycles, one latent and the other lytic. Lytic gene expression can be induced by the treatment of latently infected cells with chemical agents such as 12-O-tetradecanoylphorbol-13-acetate (TPA), sodium butyrate (Arvanitakis et al., 1996; Miller et al., 1997). It has been demonstrated that two KSHV-encoded chemokines, K6 (which encodes a vMIP-I) and K4 (which encodes a vMIP-II), are expressed in the course of lytic infection (Moore et al., 1996; Sun et al., 1999). Previous reports showed that both vMIP-I and vMIP-II induced Ca^{2+} signal transduction via certain chemokine receptors and the receptor-dependent migration of cells (Benelli et al., 2000; Chen et al., 1998; Endres et al., 1999; Kledal et al., 1997). In addition, in a chick chorioallantoic membrane assay, the both proteins showed strong angiogenic properties (Boshoff et al., 1997). However, little is known about the contribution of vMIPs to KSHV malignancy under physiologic conditions.

In this report, we generated new monoclonal antibodies against vMIP-I and vMIP-II, and confirmed the detection of both vMIP-I and vMIP-II in histological sections of tissues from MCD patients as well as in KSHV-infected PEL cell lines. In cases of KS, vMIP-II was detected, but not vMIP-I. These results suggest that the expression properties of vMIP-I and vMIP-II might be related to KSHV-associated diseases, and may even be involved in the generation of diseases. Thus, antiviral chemokine MAbs could potentially become useful tools for the diagnosis of KSHV-related diseases.

Materials and methods

Cells

Kaposi's sarcoma-associated herpesvirus-positive cell lines (BC-1, BC-3, BCBL-1 and TY-1 cells) and a negative cell line (BJAB cells) were

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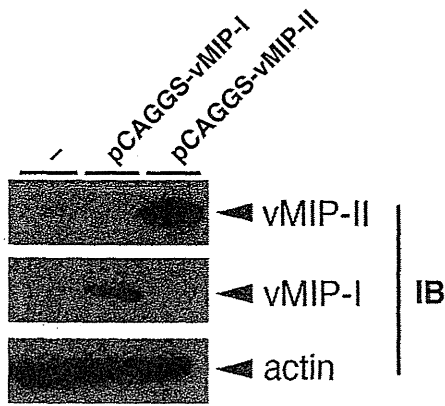

Fig. 1. Cross reactivity between anti-vMIP-I and anti-vMIP-II MAb. 293 T cells were transfected with either 2 μg of pCAGGS- vMIP-I or 2 μg of pCAGGS-vMIP-II plasmids. Forty-eight hours after transfection, the cells were harvested and expression of vMIP-I or vMIP-II was tested by Western blot analysis using the anti-vMIP-I or -vMIP-II MAb, respectively. Actin was also probed with anti-actin monoclonal Ab as a loading control.

obtained from the American Type Culture Collection (ATCC) (Manassas, VA). These cells were grown in RPMI 1640 (Nakalai Tesque, Inc., Kyoto, Japan) supplemented with 10 IU/ml penicillin G, 10 μg/ml streptomycin, 10% heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT) in a 5% CO_2 atmosphere. In addition, 293 T and 293/EBNA (Clontech) cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Nakalai Tesque, Inc.) supplemented with 10 IU/ml penicillin G, and 10 μg/ml streptomycin, 10% FBS, and 200 mM L-glutamine.

Plasmids

In order to express vMIP-I and vMIP-II, the ORFs were cloned into the pCAGGS eukaryotic expression vector, and pCAGGS-vMIP-I and pCAGGS-vMIP-II were established. The plasmid vector, pCAGGS was kindly provided by Dr. J. Miyazaki of Osaka University (Niwa et al., 1991). Briefly, fragments including vMIP-I and vMIP-II ORFs were amplified by PCR using the following primer sets: vMIP-I-Met (5′-CGGTACCGAATTCTCCAGATGGCC-3′) and vMIP-I-Ter (5′-ACTCGA-GAATTCTACTTGTCATCGTCGTCCTTGTAGTCGGAAGCTATGGCAGGCAG-3′); and vMIP-II-Met (5′-AGGTACCGAATTCAGTTATGGACACCAAGGGC-3′) and vMIP-II-Ter (5′-ACTCGAGAATTCTACTTGTCATCGTCGTCCTTG-TAGTCGGAGCGAGCAGTGACTGG-3′). The PCR products were cloned into pCR2.1 (Invitrogen) and sequenced. After digestion with *Eco*RI, the fragments were ligated into the *Eco*RI site of the pCAGGS vector. Then, the DNA fragments encoding vMIP-I and vMIP-II were liberated by *Eco*RI, and were inserted into pCAGGS to generate the expression vectors pCAGGS-vMIP-I and -vMIP-II, respectively. vMIP-I (pGEX-vMIP-I) and vMIP-II (pGEX-vMIP-II) were also generated using PCR-based technology using BCBL-1 genomic DNA as a templete. The coding region, without a signal peptide, was amplified with vMIP-I-Eco (CAGAATTCGCGGGGTCACTCGTGTCG-3′), vMIP-I-Sal (CTGTCGACCGTC-TAAGCTATGGCAGG-3′), vMIP-II-Eco (5′-CGGAATTCGCGTCCTGGCATA-GACCG-3′), and vMIP-II-Sal (5′-GGGTCGACATTCTTCAGCGAGCAGTG-3′). The amplified vMIP-I and the vMIP-II fragments were digested with *Eco*RI and *Sal*I and inserted downstream of the GST coding of pGEX-5X-1 (GE Healthcare, Uppsala, Sweden) at the *Eco*RI and *Sal*I sites to construct pGEX-vMIP-I and pGEX-vMIP-II. To express a full-length and the deletion mutants of the GST-vMIP-I and GST-vMIP-II fusion protein, the genes for GvM1-Full, GvM1-D1, GvM1-D2,

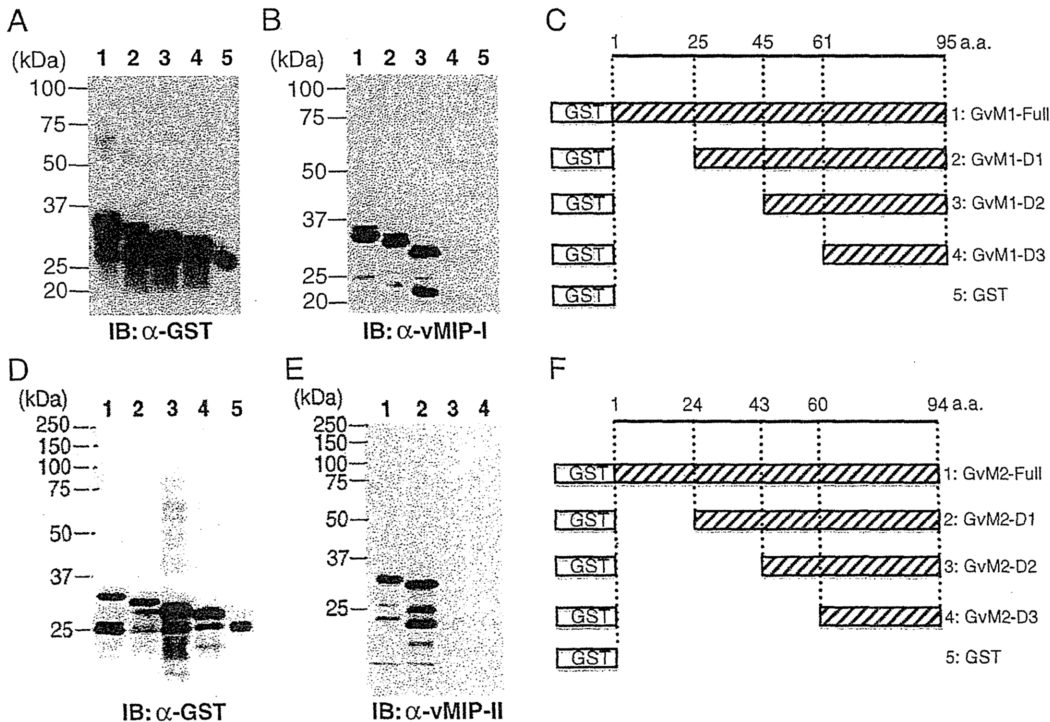

Fig. 2. Epitope mapping of the anti-vMIP-I and the anti-vMIP-II MAbs. To map the regions of vMIP-I and vMIP-II recognized by the anti-vMIP-I and anti-vMIP-II antibody, a series of GST-vMIP-I and GST-vMIP-II fusion proteins containing the individual regions of vMIP-I and vMIP-II were constructed as described in Fig. 2C and F, and the proteins were expressed in *E. coli*. The lysates of the fusion proteins, vMIP-I and vMIP-II, and its deletion mutants were immunoblotted with an anti-GST antibody (A and D) and an anti-vMIP-I (B) and an anti-vMIP-II antibody (E) to detect GST-vMIP-I or GST-vMIP-II fusion proteins. Lane 1, GvM1-Full; lane 2, GvM1-D1; lane 3, GvM1-D2; lane 4, GvM1-D3; lane 5, GvM1-D4; lan3 6, GST in Fig. 2A and B. Lane 1, GvM2-Full; lane 2, GvM2-D1; lane 3, GvM2-D2; lane 4, GvM2-D3; lane 5, GST (in D only) in Fig. 2D and E. Summary of GST-vMIP-I (C) and GST-vMIP-II (F) deletion mutants. Individual regions of vMIP-I and vMIP-II were cloned in-frame into the pGEX-5X-1 vector to generate GST-vMIP-I and GST-vMIP-II fusion proteins, respectively. The boxes at left indicate GST, and the white boxes with slashed lines indicate individual domains of vMIP-I and vMIP-II. 1, GvM1-Full(1-95a.a.); 2, GvM1-D1(25-95a.a.); 3, GvM1-D2(45-95a.a.); 4, GvM1-D3(61-95a.a.) in Fig. 2C, and 1, GvM2-Full(1-94a.a.); 2, GvM2-D1(24-94a.a.); 3, GvM2-D2(43-94a.a.); 4, GvM2-D3(60-94a.a.) in Fig. 2F.

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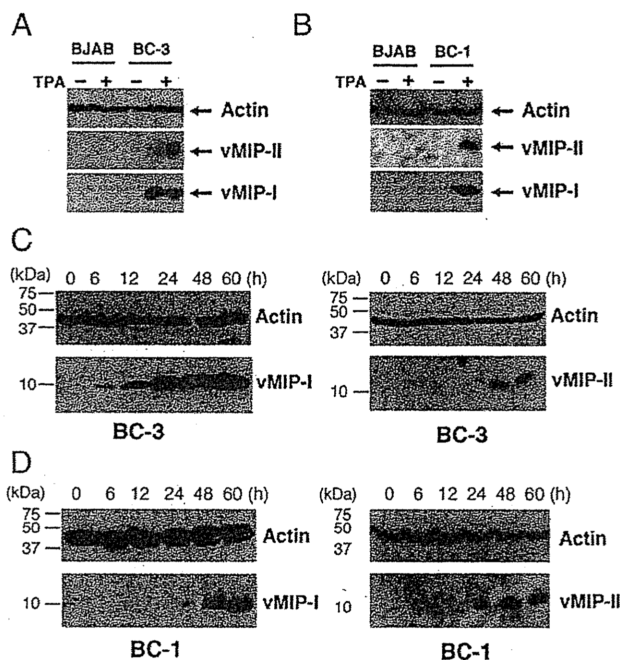

Fig. 3. Detection of vMIP-I and vMIP-II gene products in a KSHV-infected PEL cell line. BC-1 and BC-3 cells were treated with TPA for the indicated number of hours, and the whole-cell extract was prepared after the indicated time post-induction. vMIP-I and vMIP-II were detected by Western blotting and IFA with anti-vMIP-I and -vMIP-II antibodies. Western blot analysis of protein extracted from BC-3 and BJAB cells (A), and BC-1 and BJAB cells (B) with either the anti-vMIP-I or the anti-vMIP-II MAb. Arrows indicate actin, vMIP-I, and vMIP-II proteins. As expected, the estimated sizes of the vMIP-I and vMIP-II proteins, based on comparisons with the migration of molecular size markers, was around 10 kDa. Expression kinetics of vMIP-I (left panel) and vMIP-II (right panel) in TPA-treated BC-3 (C) and BC-1 (D) cells by Western blot analysis. BC-1 and BC-3 cells were harvested after 6, 12, 24, 48, and 60 h post-induction. The lysate was subjected to Western blot analysis as in (A).

GvM1-D3, GvM2-Full, GvM2-D1, GvM2-D2, and GvM2-D3 genes were generated by PCR using the following primer sets: vMIP-I-1F (5′-ATGAATTCCAGATGGCCCCCGTCCAC-3′) and vMIP-I-5R (5′-CCGTGTCGACCGTCTAAGCTATGGCAGGCAGC-3′); vMIP-I-2F (5′-ATGAATTCGCGGGGTCACTCGTGTCG-3′) and vMIP-I-5R; vMIP-I-3F (5′-ATGAATTCCCGCCCGTCCAAATTC-3′) and vMIP-I-5R; vMIP-I-4F (5′-ATGAATTCCCAAAACCCGGAGTTATTTTGC-3′) and vMIP-I-5R; vMIP-II-1F (5′-CGGAATTCGTTATGGACACCAAGGGC-3′) and vMIP-II-5R (5′-GGCAGTCGACTCTTCAGCGAGCAGTGACTG-3′); vMIP-II-2F (5′-GGGAATTCCTGGGAGCGTCCTGGCATAGAC-3′) and vMIP-II-5R; vMIP-II-3F (5′- AAGAATTCTTACCACAGGTGCTTCTGTCC-3′) and vMIP-II-5R; and vMIP-II-4F (5′-TGGAATTCAAGCCGGGTGTGATATTTTTG-3′) and vMIP-II-5R. The PCR products were cloned into pCR2.1 (Invitrogen, Carlsbad, CA) and confirmed by sequencing. The products were digested with the *Eco*RI and *Sal*I restriction enzymes and were cloned into pGEX-5X-1 (GE Healthcare). The PCR conditions for all products were as follows: 25 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min in a TP480 PCR thermal cycler (Takara Shuzo, Kyoto, Japan).

Immunization and generation of monoclonal Abs against vMIP-I and vMIP-II

In mice, anti-vMIP-I and -vMIP-II antibodies were raised against the GST-vMIP-I and GST-vMIP-II fusion protein, respectively. These GST fusion proteins were purified on a glutathione-Sepharose 4B column (GE Healthcare), and the GST-vMIP-I and the GST-vMIP-II fusion proteins were conjugated to keyhole limpet hemocyanin KLH (Calbiochem. Co., La Jolla, CA). Mice were initially immunized with 250 μg each of the purified GST-vMIP-I or -II fusion protein in Freund's complete adjuvant administered to the peritoneal cavity, and 200 μg of the antigen in Freund's incomplete adjuvant were injected again 14 and 28 days after the first injection. The mice were exsanguinated 7 days after the last injection. To generate MAbs against vMIP-I and vMIP-II, hybridomas were established by fusing splenocytes from the hyperimmune mice using a nonproducing myeloma cell line, Sp-2/0-Ag14 (ATCC, Manassas, VA). After selection in medium containing hypoxanthine-aminopterin-thymidine, cells secreting MAbs were screened by immunofluorescence assays (IFA). The TPA-induced and -uninduced BCBL-1 cells were fixed in acetone and exposed to supernatants of the hybrid cells. Clones secreting antibodies reactive with TPA-stimulated BCBL-1 cells were expanded and isolated by limiting dilutions.

Transfection analysis of vMIP-I and vMIP-II

To express the vMIP-I and vMIP-II proteins, 293/EBNA cells were transfected with pCAGGS-vMIP-I and -vMIP-II plasmids using TransIT-LT1 (Mirus Bio LLC, Madison, WI). The transfected cells were incubated for 48 h in DMEM supplemented with 10% FCS. The cells were harvested and lysed with lysis buffer (0.05 M Tris-HCl [pH 8.0], 0.15 M NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% sodium-dodecyl sulfate [SDS]). The cell lysate was fractionated by electrophoresis on 16% polyacrylamide gel as described below.

Antibodies and Western blotting

The expression of vMIP-I and vMIP-II in BC-3 cells stimulated with TPA was determined with MAbs against vMIP-I and vMIP-II, respectively, as noted above. The concentration of proteins extracted from BC-3 cells was normalized using a BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL). The samples were subjected to SDS-15% polyacrylamide gel electrophoresis under reducing conditions, and were electrophoretically transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were blocked for 1 h while being shaken at room temperature in PBS containing 0.05% Tween 20 and 5% w/v nonfat skim milk. The membranes were incubated with a primary antibody and were then incubated for 1 h with an appropriate dilution of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA). The primary antibody against actin, anti-actin (Ab-1) mouse MAb, was purchased from Merck (Merck KGaA, Darmstadt, Germany). The bound HRP-labeled antibodies were detected with a West Pico substrate kit for horseradish peroxidase (Thermo Fisher Scientific Inc).

IFA

BC-3 cells (10^7 cells) in RPMI 1640 medium with supplements were induced with 25 ng/ml TPA (Sigma Chemical Co., St. Louis, MO). The cells were collected after 0, 4, 8, 12, 24, 48, and 60 h for analysis of the expression kinetics, and for cellular localization analysis 48 h after exposure to TPA. The cells were washed in phosphate-buffered saline (PBS), pH 7.4, and spotted on glass slides. The spots were air-dried, then fixed in ice-cold acetone for 10 min. The cells were then washed with a washing buffer (PBS supplemented with 0.1% Triton X-100) for 15 min, and incubated with either an anti-vMIP-I or an anti-vMIP-II MAb (diluted 1:100 in IFA dilution buffer [PBS containing 2% bovine serum albumin, 0.2% Tween-20, and 0.05% NaN_3]) for 1 h at 37 °C. Then, the slides were washed with the washing buffer, and incubated for 1 h at room temperature with a pre-standardized diluted fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Tago Immunologicals, Camarillo, CA). The slides were washed and stained with 4′, 6′-diamidino-2-phenylindole (DAPI) to detect nuclei and were mounted with 50% (v/v) glycerol in PBS. For formalin-fixed paraffin-embedded tissues, antigen retrievals were performed on the deparaffined sections using citrate buffer. Alexa 488 or 568-conjugated

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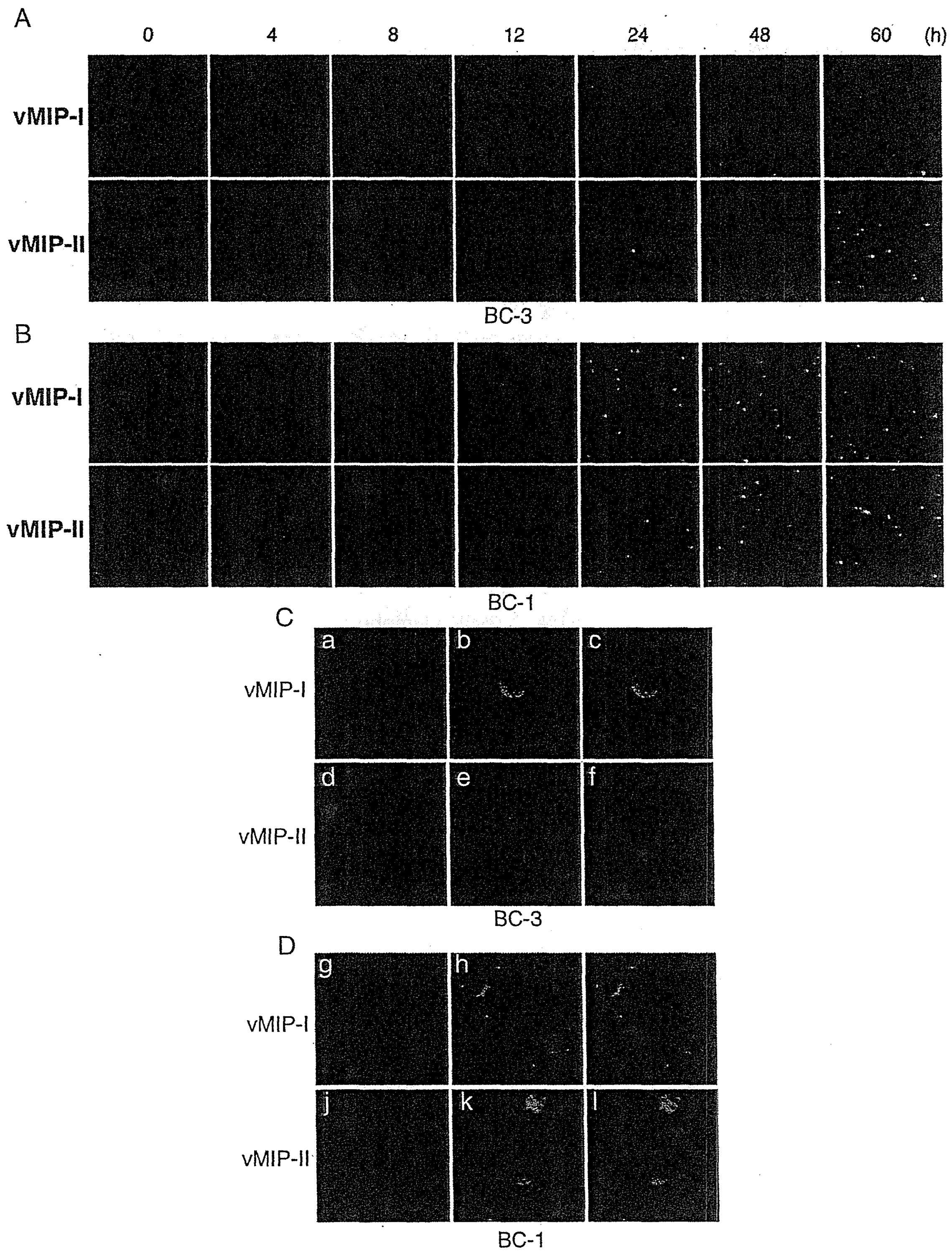

Fig. 4. Expression of vMIP-I and vMIP-II in BC-3 and BC-1 cells by IFA. After 4, 8, 12, 24, 48, and 60 h, BC-3 (A) and BC-1 (B) cells were labeled either with the anti-vMIP-I (upper) or the anti-vMIP-II (lower) MAb followed by goat anti-mouse FITC-conjugated Abs. FITC photomicrographs showing anti-vMIP-I and anti-vMIP-II immunoreactivity in BC-3 and BC-1 cells treated with TPA. (C) Cellular localization of vMIP-I and vMIP-II in BC-3 (C) and BC-1 (D) cells. The cells were stained with DAPI (a, d, g and j), and the localization of vMIP-I and vMIP-II was visualized by IFA with anti-vMIP-I or -vMIP-II MAbs (b, e, h and k); panel a and b, d and e, g and h, and j and k were merged (c, f, i and l). Fluorescence photomicrographs revealed anti-vMIP-I and -vMIP-II immunoreactivity using FITC-conjugated anti-mouse IgG MAb.

anti-mouse or rabbit antibodies (Invitrogen) were used as the secondary antibodies. Confocal microscopic analysis was performed (FV-1000, Olympus, Tokyo, Japan), and the contrast was adjusted before the images were exported as TIFF files to Adobe Photoshop.

Immunohistochemistry

Formalin-fixed paraffin-embedded tissues from KS and MCD patients, and those from an animal model of KSHV-associated solid lymphoma

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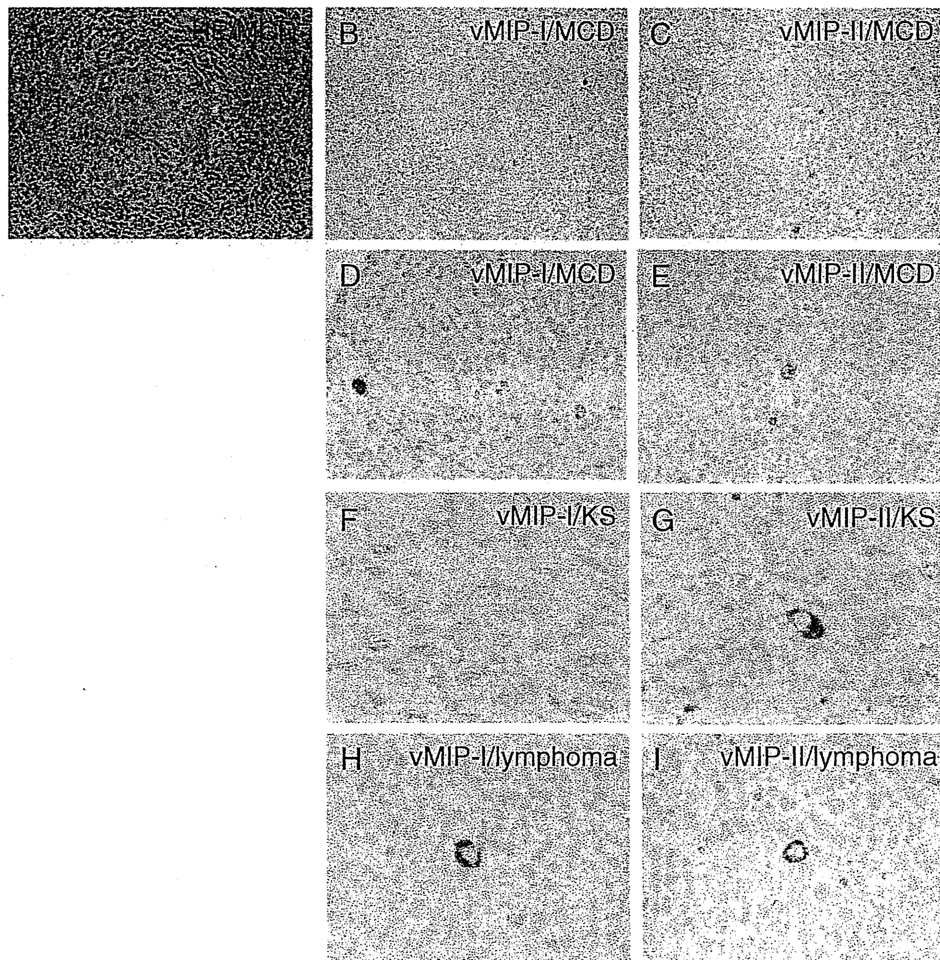

Fig. 5. Expression of vMIP proteins in KSHV-associated diseases. (A–C) Hematoxylin and eosin staining and immunohistochemistry for vMIPs in serial sections of a tissue sample from a patient with MCD. Brown stains indicate positive signals. The nucleus was counter-stained by hematoxylin. (D and E) Higher magnification view of vMIPs expression in an MCD case. Some large lymphocytes in the mantle zone were stained. (F and G) vMIP-I and vMIP-II expression in a KS sample. (H and I) Expression of vMIPs in an animal model of KSHV-associated lymphoma in SCID mice.

were sectioned and stained with hematoxylin and eosin (H&E). Immunohistochemistry of the serial sections was performed with either the anti-vMIP-I or -II MAb. For the second- and third- phase reagents used for immunostaining, a CSAII kit (DAKO, Copenhagen, Denmark) was used. An animal model of KSHV-associated solid lymphoma, which was established as described previously (Katano et al., 2000b), was also subjected to immunohistochemical analysis. Briefly, TY-1 cells were inoculated into the subcutaneous tissue of mice with severe combined immunodeficiency (SCID). One month after inoculation, lymphomas appeared in the subcutaneous region at the inoculation site. Lymphoma cells contained the KSHV genome, and expressed various viral proteins of KSHV (Katano et al., 2000b).

Table 1
Expression of vMIP-I and vMIP-II in MCD and KS tissue samples.

	KSHV proteins, (+)/total	
Cases	vMIP-I	vMIP-II
MCD	(3)/3	(3)/3
KS	(0)/5	(2)/8

Chemotaxis assays

Chemotaxis assays were performed as described previously (Nakano et al., 2003). Briefly, THP-1 cells were washed twice with chemotaxis buffer, 0.5% bovine serum albumin, 20 mM HEPES, pH 7.4, in RPMI 1640. Migration of cells was assessed in a cell culture chamber (Costar, Cambridge, MA), with the upper and lower compartments separated by a 3 μm pore size polycarbonate filter (???). The lower compartment of the chamber was filled with dilutions of vMIP-I, vMIP-II (R&D Systems, Minneapolis, MN) or with PBS alone, and/or with each 10 μg/ml anti-vMIP-I or -vMIP-II MAbs at a volume of 600 μl. The upper compartment contained 100 μl of THP-1 cell suspensions in chemotaxis buffer (10^5 cells/well). The chambers were then incubated for 4 hours at 37 °C, 5% CO_2, and spun at 300 x g, 4 °C, for 5 min. Finally, the cells from the lower compartment were counted.

Results

Specificity of the anti-vMIP-I MAb and the anti-vMIP-II MAb

In order to check specificity of the MAbs, we transfected vMIP-I and vMIP-II expression vectors (pCAGGS-vMIP-I, and -II) into 293/EBNA

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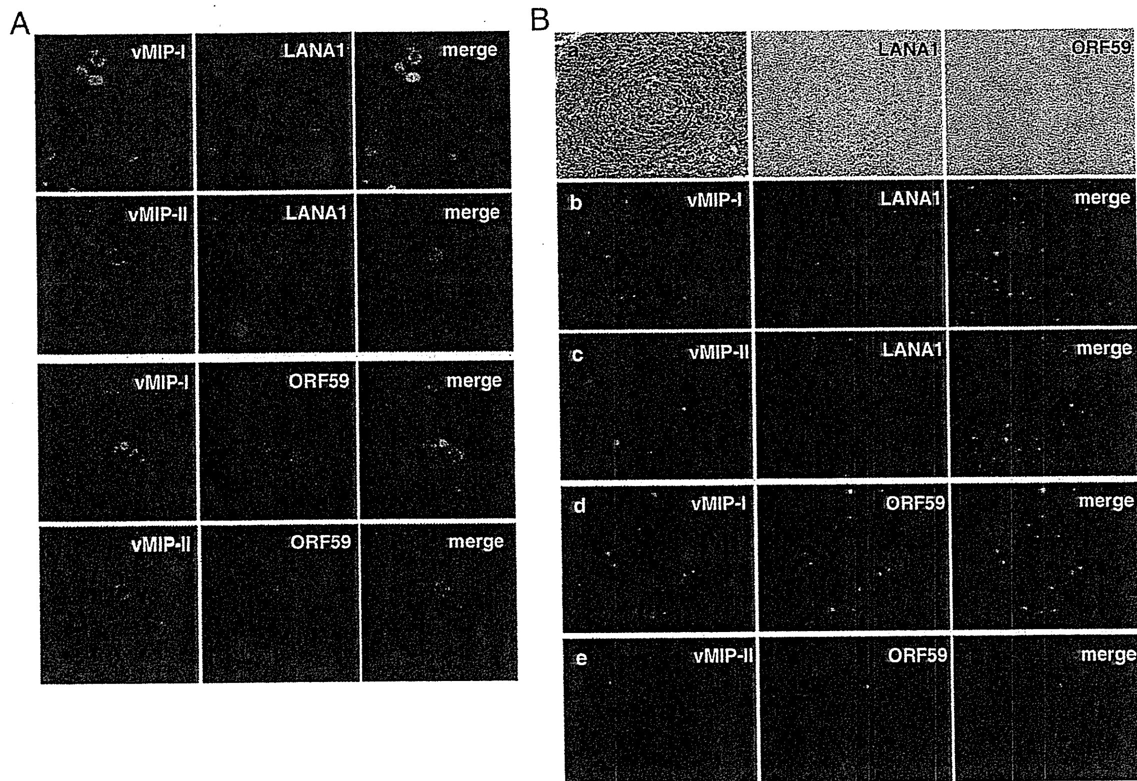

Fig. 6. (A) Expression of vMIPs. LANA1 and ORF59 in the animal model of KSHV-associated solid lymphoma by confocal microscopy. vMIPs were labeled with Alexa 488 (green). LANA1 (upper panels) and ORF59 (lower panels) were labeled with Alexa 568 (red). (B) Expression of vMIPs in MCD. (a) HE staining and immunohistochemistry of LANA1 and ORF59. (b–e) Immunofluorescence assay on MCD lesion. A germinal center is shown in the center of each panel. This case is KSHV-positive large B cell lymphoma arising in MCD.

cells, respectively. The total lysate of the transfected cells was subjected to Western blot analysis. vMIP-I and vMIP-II proteins were detected with anti-vMIP-I or vMIP-II MAbs, respectively (Fig. 1). These antibodies did not show cross-reactivity each other.

Epitope mapping of the anti-vMIP-I and anti-vMIP-II MAbs

We established hybridoma clones secreting MAbs against vMIP-I and vMIP-II, respectively. To map the regions of vMIP-I and vMIP-II where anti-vMIP-I and anti-vMIP-II antibody reacted, a series of GST-fused vMIP-I and vMIP-II deleted proteins were constructed as described in Fig. 2C and F, and used for Western blot analysis with an anti-GST antibody (Santa Cruz Biotechnology Inc), (Fig. 1A, D) and the anti-vMIP-I or the anti-vMIP-II (Fig. 1B, E) antibody, respectively. The results showed that all GST-vMIP-I and GST-vMIP-II fusion proteins interacted with the anti-GST antibody (Fig. 2A, D) and showed that GvM1-Full, GvM1-D1, and GvM1-D2 reacted with the anti-vMIP-I antibody, whereas GvM1-D3 did not (Fig. 1B), and GvM2-Full and GvM2-D1 reacted with the anti-vMIP-II antibody, whereas GvM2-D2, and GvM2-D3 did not (Fig. 2E). Thus, these results demonstrated that an anti-vMIP-I MAbs was successfully generated and suggest that the amino acid residues 61 to 95 of vMIP-I could be a major epitope reacted with the anti-vMIP-I antibody. On the other hand, the amino acid residues 24 to 42 of vMIP-II could be an epitope reacted with the anti-vMIP-II antibody.

Expression of vMIP-I and vMIP-II in the KSHV-infected PEL cell line

We tested vMIP-I and vMIP-II expression in KSHV and Epstein Barr virus (EBV) dually infected PEL cell lines (BC-1), KSHV infected PEL cell lines (BC-3) and in non-infected Burkitt's lymphoma cell line (BJAB), and detected them in TPA-stimulated BC-3 and BC-1 cells with developed antibodies, but not in BJAB cells non-stimulated BC-3 or BC-1 cells (Fig. 2A, B). In a KSHV infected PEL cells, BC-1 and BC-3, vMIP-I and vMIP-II were detected around at 10 kDa, which matches the size deduced from amino acids length (Fig. 3C, D). Actually, vMIP-I was detected from 6 hours post induction and vMIP-II was at 24 hours in BC-3 cells (Fig. 3C), and vMIP-I and vMIP-II were detected at 24 h in BC-1 cells (Fig. 3D). In the immunofluorescence microscopy, the number of vMIP-II expressing cells seemed to be more than that of vMIP-I in BC-3 cells (Fig. 4A, B). In order to analyze the cellular localization of vMIP-I and vMIP-II protein, BC-3 and BC-1 cells stimulated with TPA were doubly labeled with DAPI (Fig. 4C, a, d and D, g, j), and either the anti-vMIP-I MAb (Fig. 4C, b and D, h) or the anti-vMIP-II MAb (Fig. 4C, e and D, k). Merged images were shown in Fig. 4C, c, f, and D, i, l). The vMIP-I and the vMIP-II clearly showed cytoplasm and possibly membranes in TPA-induced BC-3 and BC-1 cells (Fig. 4C, b, e, and D, h, k).

Expression of vMIPs in KSHV-associated diseases

To know the expression of vMIPs in KSHV-associated diseases, immunohistochemistry for vMIPs was performed on pathological samples of eight KS cases, three MCD cases, and the animal model of KSHV-associated solid lymphoma (Fig. 5). Immunohistochemistry demonstrated that vMIP-I and vMIP-II were detected in some cells in the mantle zone of germinal center and the interfollicular zone in KSHV-positive MCD samples (Fig. 5A to E). Both vMIP-I and vMIP-II were detected predominantly in the cytoplasm of large lymphocytes. The numbers of positive cells varied among three MCD cases examined. On the other

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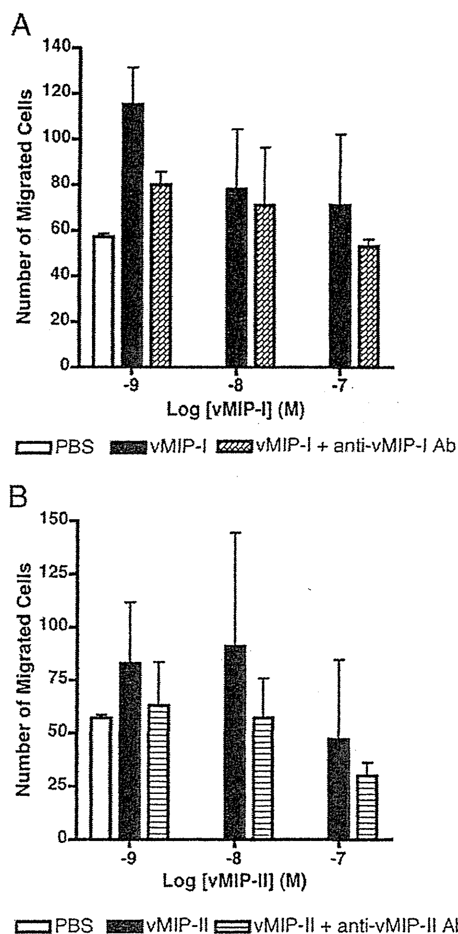

Fig. 7. Neutralizing activity of anti-vMIP-I and -vMIP-II MAbs. THP-1 cell migration in response to increased concentrations of vMIP-I and vMIP-II (1, 10, 100 nM), and the neutralizing activity of 10 μg/ml anti-vMIP-I and -vMIP-II MAbs against vMIP-I and vMIP-II were measured, as outlined in Materials and Methods, by using the transwell migration assay system. Various doses of vMIP-I and vMIP-II were tested for their ability to induce the chemotaxis of THP-1 cells. The data presented are from one experiment, and are representative of the triplicate experiments performed. The error bars indicate the standard deviations of three independent experiments.

hand, any positive signal of vMIP-I was not observed in all KS cases (Fig. 5F, G). vMIP-II was rarely detected in the cytoplasm of spindle cells in two KS cases at the nodular stage out of eight KS cases. In the samples of animal model of KSHV-associated solid lymphoma, both vMIP-I and vMIP-II were detected in the cytoplasm of a part of lymphoma cells (Fig. 5H, I). These data showed that vMIP-I and vMIP-II were expressed in cells in MCD and KSHV-associated lymphoma, but vMIP-II was rarely in KS (Table 1). To know the association of vMIPs expression with expression of other KSHV-encoded proteins, we examined immunofluorescence assay on KSHV-associated diseases. Since, all KSHV-infected cells express LANA1, vMIPs-positive cells were positive for LANA1. However, expression pattern of LANA1 showed diffuse nuclear staining in vMIPs-positive cells in the animal model of KSHV-associated solid lymphoma (Fig. 6A). Confocal microscopy revealed that vMIP-I stain showed usually cytoplasmic pattern, but rarely diffuse nuclear staining pattern *in vivo*. Almost all cells with vMIPs expression were also positive for ORF59 protein, a lytic protein of KSHV. IFA also demonstrated that vMIPs-positive cells expressed LANA1 at various levels in MCD clinical samples (Fig. 6B, a to c). A large portion of vMIPs-positive cells also expressed ORF59 protein in MCD (Fig. 6B, d, e). These data suggest that vMIPs are expressed by cells with KSHV-lytic infection in KSHV-associated MCD and lymphoma.

Neutralization of vMIP-I and vMIP-II by anti-vMIP-I and anti-vMIP-II MAbs

We examined whether the anti-vMIP-I and anti-vMIP-II MAbs could neutralize the chemoattractant of vMIP-I and vMIP-II to induce the migration of THP-1 cells. As expected, vMIP-I and vMIP-II induced migration of THP-1 cells (Fig. 7A, B), but not with PBS alone. However, anti-vMIP-I and anti-vMIP-II MAbs inhibited respective vMIP-I and vMIP-II-induced cell migration of THP-1 cells at 10 μg/ml final concentration.

Discussions

It was known that KSHV encodes three chemokine genes of the so-called viral macrophage inflammatory proteins: *vMIP-I*, *vMIP-II*, and *vMIP-III* in the genome. Analysis of the translated amino acid sequence indicate that the vMIP-I and vMIP-II gene have four conserved cysteines capable of forming two essential disulfide bonds (first cysteine and third cysteine, and second cysteine and fourth cysteine). The family of chemokines comprises CC, CXC, C, and CX_3C subfamilies. The vMIP-I and vMIP-II have four cysteines, the first two of which are found in the sequence of CC, which correspond to the CC profile. These gene products were expressed in the phase of KSHV lytic infection (Moore et al., 1996; Sun et al., 1999). Both vMIP-I and vMIP-II were expressed in a KSHV-infected cell lines, BC-3, which had been treated with TPA. Mono-specific polyclonal Abs against vMIP-I and vMIP-II have been described in previous studies that investigated the localization of vMIPs in PEL cells (Nakano et al., 2003). In the present study, we developed the respective MAbs that reacted either with KSHV vMIP-I or vMIP-II. We first applied these MAbs against KSHV vMIP-I and vMIP-II to detect KSHV-infected BC-3 and BC-1 cells by Western blotting and immunofluorescence assay. The Western blot analysis revealed that both the anti-vMIP-I and the anti-vMIP-II MAbs reacted to the 10-kDa proteins considered specific to the respective vMIP protein. The anti-vMIP-I MAb was shown to be reactive with the epitopes in the middle of the protein (sequence, PPVQILKEWYPTSPAC), and the epitope of the anti-vMIP-II MAb was shown to be reactive at the N-terminal end (sequence, LGASWHRPDKCCLGYQKRP). Further immunofluorescence analysis of the cellular localization of both vMIP-I and vMIP-II with anti-vMIP-I and anti-vMIP-II MAb showed a cytoplasmic pattern of expression in BC-3 and BC-1 cells. As the results indicated that these gene products were expressed in the cytoplasm, it might be located at the KSHV-infected BC-3 or BC-1 cells membrane prior to secretion. An investigation of the antigenic specificities of MAbs against KSHV vMIP-I and vMIP-II in MCD and KS patients has not yet been reported. Here, immunohistochemical analysis detected only vMIP-II in samples from both KS and MCD patients, but vMIP-I was not detected in KS cases: however, both vMIP-I and vMIP-II proteins were expressed in some cells in the interfollicular zone of MCD tissues. Lytic proteins of the KSHV such as K8, RTA, and ORF59 have been detected in large lymphocytes in the mantle zone of MCD cases (Dupin et al., 1999; Katano et al., 2000a). The expression of vMIPs showed a similar pattern to that of the lytic proteins in MCD tissues. In contrast, lytic protein expression, including that of vMIPs, was rare in the KS lesions (Abe et al., 2006). In the present study, we demonstrated that vMIPs were expressed in the cells expressing ORF59 protein. Thus, our data clearly indicated that the expression of vMIPs is associated with lytic infection in individual cells affected by KSHV-associated diseases. Human monocytic cell line THP-1 respond to various chemokines suggesting that they express receptors for these chemokines (Wang et al., 1993). Previous study, vMIP-I and vMIP-II were shown chemotaxis in THP-1 cells (Nakano et al., 2003). It has been reported that vMIP-I acts as a specific agonist for CC chemokine receptor 8 (CCR8) (Dairaghi et al., 1999; Endres et al., 1999) and vMIP-II shows a Ca^{2+} flux as a specific agonist for CCR3 (Boshoff et al., 1997). Our data showed anti-vMIP-I and anti-vMIP-II MAbs were able to neutralize vMIP-I- and vMIP-II-mediated chemotaxis in THP-1 cells. However, neutralizing activities

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of anti-vMIP-I MAb was apparently low, even the addition of 10 μg/ml MAbs. These findings support the assumption that anti-vMIP-I and -vMIP-II MAbs-blocked chemotaxis in THP-1 cells act through binding to the certain amino acid residue of vMIP-I and vMIP-II.

In summary, MAbs developed specifically for this series were used to detect vMIP-I and vMIP-II in MCD and KS tissues, which may account for certain clinical features of MCD and KS. To gain a better understanding of these important viral genes, additional studies will be needed that focus on revealing vMIP-I and vMIP-II expression profiles during lytic infection. Taken together, these studies provide an insight into the pathogenesis of the contribution of vMIP-I and vMIP-II to the lytic induction of KSHV. These MAbs could serve as useful tools to clarify the pathogenesis of KSHV-related diseases.

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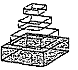

For the future studies of Kaposi's sarcoma-associated herpesvirus

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It is 18 years since Kaposi's sarcoma-associated virus (KSHV), also called human herpesvirus 8 (HHV-8), was found from Kaposis' sarcoma (KS) by Chang et al. (1994). More than 8, 000 reports have been published so far and we have learned many things from this virus. I would like to say it is about time to look back previous studies and to think what to study next on the virus, and planed a topic to think what to study next on the virus for future.

Herpesviruses have relatively big genomes and encode a 100 genes or so. Thus, the virion assembly/structure, gene expression regulation and attachment/entry are complicated and have known only an iceberg of them. Studying the details how the viruses run their life cycles and cause diseases in their processes will lead to exploring new therapeutic drugs/methods.

A viral life cycle starts from attachment on the susceptible cells and then, entry into the cells, followed by the viral gene expression, the genome replication, the particle assembly and finally the daughter viruses egress out of the cells. This process is skillfully built and all the viral genes are required for the process, though there are essential genes and non-essential ones. Viral pathogenesis could be established during this process by the interaction between viruses and host cells, and individual host systems such as immune system. In this topic, although I would like to cover all the processes, thankfully, 15 specialists in each field have contributed for this topic.

Polizzotto et al. (2012) described clinical manifestations of KSHV-associated diseases. So far, there were few reports on clinical manifestations of primary KHSV infection. In this term, KSHV inflammatory cytokine syndrome (KICS) is a new concept and we might have been looked over an important disease sign on KHSV infection. We will have to be more careful about what happens in primary KSHV infection than before.

Fukumoto et al. (2011) describe KSHV infection from a pathologist's points of view. Pathologic study is very important to know what happens in the lesions. Currently, we are able to know what is going on only in the KSHV associated lesions such as Kaposi's sarcoma, multicentric Castleman's disease and primary effusion lymphomas (PEL) of human samples suffered from KSHV infection, but once an infection model is established, chronological pathologic studies will provide a lot of information on how KSHV-associated diseases are formed.

Chakraborty et al. (2012) review the entry mechanism of KSHV into cells. In general, herpesviruses can infect various kinds of cells *in vitro* including non-human cell lines, but the infectivity to B lymphocyte originated cells is very inefficient. Their report will give us a hint why such phenomenon happens.

An immediate early gene, *RTA* (reactivation and transcription activator) is very important for the viral lytic replication induction and shows multifunctions. We still have not understood how the factor functions. Guito and Lukac (2012) and Tsai et al. (2012) review or report mechanistic regulation of this strong transactivator, respectively.

Jackson et al. (2012) describe ORF57, which is also an interesting and multifunctional protein. This is involved in post-translational processes of the viral gene expression as sumoylation and ubiquitination described by Campbell and Izumiya (2012) and Ashizawa et al. (2012) respectively. We had believed that K-bZIP, a homolog of Epstein-Barr virus Zta was a transactivator and origin recognition factor in the lytic replication. K-bZIP, however, has other important roles for KSHV lytic replication. In latency, metabolism of LANA (latency-associated nuclear antigen) could be critical for KSHV-induced tumor formation and/or its phenotype.

Viral particle assembly is virologically an exciting and interesting field. There have been few reports on this, Sathish et al. (2012) try to search this issue.

The detail replication mechanism of KSHV in both lytic and latent phase has been still unclear. In latency, the virus is supposed to utilize host replication machinery including pre-replication complexes (pre-RC) for the viral replication initiation in the presence of LANA. The viral factor, LANA, is an essential factor, but its necessity has not been elucidated well. LANA binds with LANA-biding sites (LBS) and recruits origin recognition complexes (ORCs) on the viral replication origin (ori-P), which cannot account for necessity of the GC-rich element followed by LBS. Ohsaki and Ueda (2012) will give us a hint about this question.

Viral immune evasion system is very tactic to maintain its latency in case of herpesviruses. The maintenance of latency is then critical for the virus to wait for reactivation to produce daughter viruses, whose transition may a step for the viral oncogenic process. Lee et al. (2012) summarize KSHV immune evasion strategy and make a comment on the future landscape.

Kaposi's sarcoma-associated virus mediated tumorigenesis including PEL and KS has been still unclear, though there are many reports on individual viral putative oncogenes. KSHV has not been reported to infect and immortalize and/or transform endothelial cells or peripheral blood mononuclear cells *in vitro*. And thus, we have not known how the viral genes with oncogenic potentials such as *vFLIP*, *vCYC*, *vGPCR* and so on in addition to *LANA* cooperate in the viral oncogenic process. DiMaio and Lagunoff (2012) address on this issue and look forward for this field.