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Kaposi´s sarcoma-associated herpesvirus ORF36 protein induces chromosome condensation and phosphorylation of histone H3

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Summary. – Kaposi´s sarcoma-associated herpesvirus (KSHV) has been known as an agent causing Kaposi´s sarcoma, primary effusion lymphoma, and multicentric Castleman´s disease. In the lytic phase of the virus cycle, various viral genes are expressed, which causes host cell dysregulation. Among the lytic genes, viral protein kinase (vPK) encoded by ORF36 is a member of serine/threonine protein kinase (CHPK) family, which is involved in viral gene expression, viral DNA replication and encapsidation, and nuclear egress of virions. Recent studies have shown that the BGLF4 protein of Epstein-Barr virus (EBV), a member of the CHPK family, alters the host cell chromatin structure through phosphorylation of its key regulators. The role of KSHV ORF36 in cellular mitotic events, however, is not yet understood. In the current study, we showed that KSHV ORF36 induced chromosome condensation and phosphorylation of histone H3 on Ser 10, which are known as cellular mitosis markers. These processes have occurred in a kinase activity-dependent manner.

Keywords: Kaposi´s sarcoma-associated herpesvirus; viral protein kinase; ORF36; chromosome condensation; histone H3; phosphorylation

Introduction

Kaposi´s sarcoma-associated herpesvirus (KSHV), also known as gamma herpesvirus 8, has a large double-stranded DNA and two phases of the life cycle, latent and lytic. In the lytic phase, most of the viral genes are expressed for effective viral replication and virion production. Among these genes, viral protein kinase (vPK) encoded by KSHV ORF36 has a serine/threonine kinase activity, which activates host signaling pathway such as c-Jun N-terminal kinase and inhibits phosphorylation of focal adhesion kinase (FAK) (Hamza *et al*., 2004; Park *et al*., 2007). Most herpesviruses have a putative serine/threonine protein kinase, a protein termed also a conserved herpesviral protein kinase (CHPK). CHPKs include herpes simplex virus (HSV) type I UL13, varicella-zoster virus (VZV) ORF47, human cytomegalovirus (HCMV) UL97, and Epstein-Barr virus (EBV) BGLF4. CHPK proteins contain 11 conserved domains and a lysine residue in subdomain II that is essential for kinase activity (Kawaguchi and Kato, 2003; Kuny *et al*., 2010). Members of this kinase family share some conserved functions, such as hyperphosphorylation of the cellular translation factor EF-1δ, and phosphorylation of their own viral proteins (Jacob *et al*., 2010; Kawaguchi *et al*., 2003). In addition, CHPKs have been shown to be critical in viral gene expression and viral DNA replication due to their function in protein phosphorylation (Gershburg and Pagano, 2008). Recent studies have suggested that these viruses secure the space for productive viral replication in the nucleus. During HSV-1 infection, the nuclear volume is expanded, which results into enlarged extrachromosomal space (Monier *et al*., 2000). On the other hand, a previous report showed that EBV BGLF4-induced chromosome condensation via interaction with condensin and topoisomerase II (Topo II) leads to the expansion of the extrachromosomal space for viral DNA replication (Lee *et al*., 2007). According to the latter study, EBV BGLF4 interacts with condensin, which is a key component of the mitotic

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Abbreviations: CHPK = conserved herpesviral protein kinase; EBV = Epstein-Barr virus; HSV = herpes simplex virus; KSHV = Kaposi´s sarcoma-associated herpesvirus; Topo II = topoisomerase II; vPK = viral protein kinase

assembly complex and phosphorylates cdc2 consensus motifs of condensin. Although it has been shown that protein kinases encoded by herpesviruses and cdc2 protein kinase may have the same targets, a similar function of KSHV ORF36 has not yet been determined (Kawaguchi *et al*., 2003). Based on these previous studies, we speculated that KSHV ORF36 may cause structural alteration of cellular chromatin. In this study, we showed that cellular chromosome condensation was induced by transiently expressed KSHV ORF36 in a kinase activity-dependent manner. Consistent with this observation, the level of phosphorylation of histone H3 on Ser 10, which has been established as a critical step for chromosome condensation, was also increased in the presence of KSHV ORF36 protein (Hendzel *et al*., 1997; Van Hooser *et al*., 1998; de la Barre *et al*., 2000). Taken together, the results suggest that KSHV ORF36 may have a functional role in changing nuclear architecture similar to the role of EBV BGLF4.

Materials and Methods

Plasmids. The pEGFP-ORF36 vector employed in a previous study (Park *et al*., 2000) was used to confirm ORF36 expression. To construct GFP-tagged ORF36 K108A mutant-expressing plasmids, inserts were amplified from pcDNA3-HA-ORF36 (K108A) plasmids using the forward primer, 5′-CCCAAGCTTNNATGCGCTGGA AGAGAATG-3′, and the reverse primer, 5′-CCCGTCGACTC AGAAAACAAGTCCGCG-3′. Amplified products were subcloned into the *Hind*III/*Sal*I site of pEGFP empty vector.

Cell culture and transfections. 293T cells were grown in DMEM containing 10 % FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. Slide-cultured 293T cells were transfected with pEGFP, pEGFP-ORF36, or pEGFP-ORF36 (K108A), using PolyExpress™ reagents (Excellgen).

Immunofluorescence assay. Transfected 293T cells were fixed with 3.7% formaldehyde for 15 min, and permeabilized with PBS

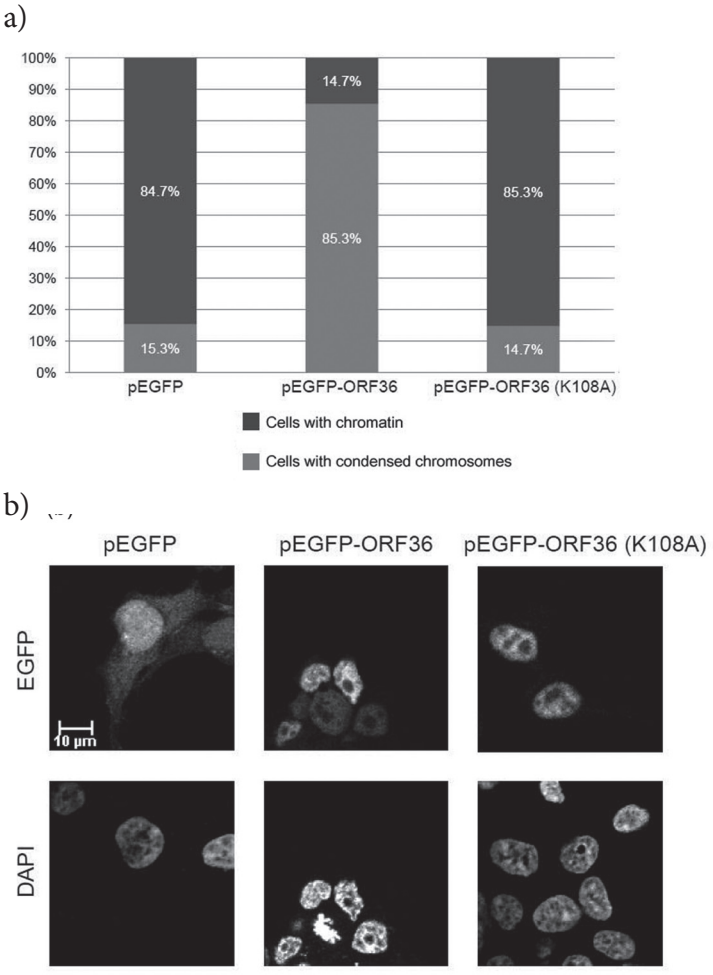

Fig. 1

KSHV vPK induces chromosome condensation

Confocal microscopy of immunostained 293T cells transfected with pEGFP, pEGFP-ORF36, or pEGFP-ORF36 (K108A). (a) Percentage of cells with condensed chromosomes. (b) Morphology of condensed chromosomes. Magnification 60x.

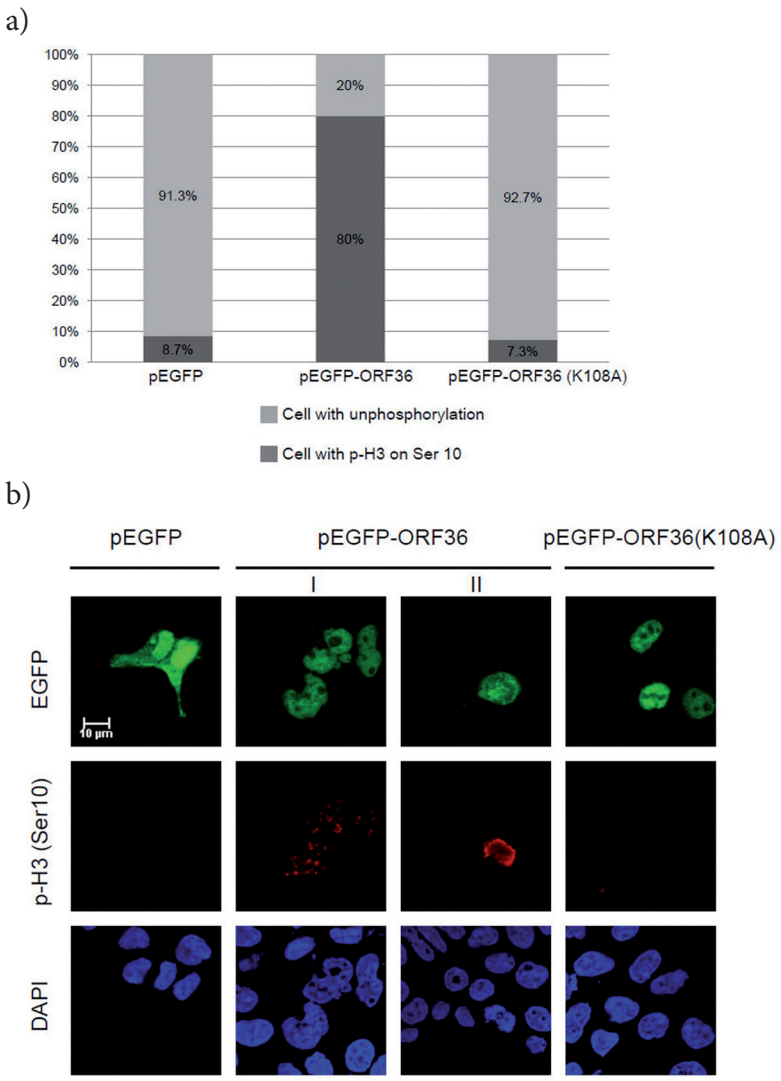

Fig. 2

KSHV vPK induces phosphorylation of histone H3 on Ser 10

Confocal microscopy of immunostained 293T cells transfected with pEGFP, pEGFP-ORF36, or pEGFP-ORF36 (K108A). (a) Percentage of cells with phosphorylated histone H3. (b) Staining pattern of cells. Magnification 60x.

containing 0.2% Triton X-100 (PBST) for 10 min on ice. To observe chromosome condensation in the nucleus, DNA was stained with 1 μg/ml of DAPI (Sigma). For immunostaining, permeabilized cells were blocked with PBST containing 1% BSA for 30 min, incubated with anti phospho-histone H3 antibody (Santa Cruz) overnight at 4°C, and washed with PBST. Next, cells were incubated with TRITC-conjugated anti-rabbit IgG antibody for 1 hr and washed with PBST. DAPI was used as a nuclear counterstain. Cells were analyzed under a confocal microscope (Nikon). In total, 150 cells were counted for a set of experiments. Experiments were performed independently at least twice.

Results and Discussion

In a previous study, EBV BGLF4 has been shown to affect host chromosome condensation via interaction with chromatin organization factors. Since viral protein kinases encoded by herpesviruses have several conserved functions, we tested the hypothesis that KSHV ORF36 protein regulates cellular chromosome condensation. To determine the function of the KSHV ORF36, we observed the morphological changes in 293T cells transiently expressing ORF36 using confocal microscope. In order to measure the ratio of transfected

cells with condensed chromosomes, we used GFP-fused ORF36 or its kinase-dead mutant, ORF36 (K108A), which has substituted the Lys to Ala at the residue 108 (Park *et al*., 2007). As shown in Fig.1b, chromosome condensation events were observed in DAPI-stained 293T cells. To compare mock- and ORF36-transfected cells, we counted cells with condensed chromosomes in 150 GFP-expressing cells. Chromosome condensation was observed in a significant percentage (>85%) of ORF36-expressing cells. In contrast, GFP-expressing cells and ORF36 (K108A)-expressing cells revealed a low level of chromosome condensation, approximately 15.3% and 14.7%, respectively (Fig. 1a,b). These results suggest that the expression of the ORF36 protein affects cellular chromosome condensation in a significant number of cells, and that the function of the ORF36 protein is dependent on the kinase activity.

To support these results, we verified the phosphorylation of the histone H3 at Ser 10, which is known to be a critical step in cellular chromosome condensation. To identify the phospho-histone H3 in the GFP- or GFP-fused ORF36-expressing cells, the phospho-histone H3 at Ser 10 was stained with TRITC-conjugated secondary antibody. As shown in Fig. 2b, we confirmed two distinctive patterns of phosphorylated histone H3. First, the majority of cells revealed dot-like staining pattern as observed in the early stage of histone H3 phosphorylation (Fig. 2b, I). Second, a proportion of cells presented a highly condensed mitotic chromosome pattern (Fig. 2b, II). The ratio of phosphorylated histone H3 in the GFP-ORF36-expressing cells is approximately 80%, while only 8.7% and 7.3%, were observed in the GFP or GFP-ORF36 (K108A) expressing cells, respectively (Fig. 2a). These results support the hypothesis that ORF36 protein induces cellular chromosome condensation via phosphorylation of histone H3 on Ser 10 in a kinase activity-dependent manner.

Histone H3 phosphorylation occurs during the late G2/M phase of the cell cycle, which is the time for the initiation of the chromosome condensation (Hendzel *et al*., 1997). Our data indicates that KSHV ORF36 protein influences this step. In the mitotic chromosome condensation, Topo II is also known as an essential factor, which regulates decatenation of chromosome DNA (Escargueil, 2001). It has been shown in a previous study that EBV BGLF4 enhances decatenation activity of Topo II, leading to the regulation of chromosome condensation (Lee *et al*., 2007). A recent study showed that Topo II is recruited to viral replication compartments during the replication process, and its importance in KSHV replication and virion production has been demonstrated using shRNA (Gonzalez-Molleda *et al*., 2012). Further studies are, therefore, required to identify the biological meaning of KSHV ORF36-induced cellular chromosome condensation in the KSHV-infected cells. In contrast to chromatin structure, EBV BGLF4 induces morphological changes of the expressing cells via rearrangement of actin filaments (Lee *et al*., 2007). Chen and colleagues also reported that EBV BGLF4 phosphorylates the cellular microtubule regulator, stathmin, and affects reorganization of the microtubule (Chen *et al*., 2010). Moreover, CHPKs have been shown to influence the redistribution of nuclear lamina and facilitate virus egression (Lee *et al*., 2007, 2008; Chen *et al*., 2010; Kuny *et al*., 2010). In view of these findings, KSHV ORF36 may also regulate some nuclear or cytoplasmic structures. Therefore, various functions of KSHV ORF36 protein shall be determined in further studies.

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