Available online at www.sciencedirect.com
SCIENCE DIRECT®
Virology 352 (2006) 100–109
VIROLOGY
www.elsevier.com/locate/yviro

Expression of Kaposi's sarcoma-associated herpesvirus-encoded K10/10.1 protein in tissues and its interaction with poly(A)-binding protein

Takayuki Kanno, Yuko Sato, Tetsutaro Sata, Harutaka Katano *

Department of Pathology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

Received 12 December 2005; returned to author for revision 6 January 2006; accepted 5 April 2006
Available online 22 May 2006

Abstract

The K10/10.1 protein is encoded by a cluster of interferon regulatory factor (IRF) homologues in the Kaposi's sarcoma-associated herpesvirus (KSHV, human herpesvirus 8, HHV-8) genome. In the present study, we showed that an anti-K10 antibody reacted with a 110-kDa protein encoded by the K10/10.1 gene of KSHV in KSHV-infected primary effusion lymphoma (PEL) cell lines. Expression of K10/10.1 protein was induced by phorbol ester in KSHV-infected cells. A reporter gene assay demonstrated that K10/10.1 protein did not influence promoter activity of human interferon genes, regardless of its homology to human IRFs. Poly(A)-binding protein (PABP) was identified as a partner of K10/10.1 protein. Immunoprecipitation revealed that K10/10.1 protein interacted with PABP specifically in PEL cell lines. IFA revealed co-localization of K10/10.1 protein and PABP in the nucleus of KSHV-infected cells. These data suggest that K10/10.1 protein may affect the translational status or stability of mRNA in host cells.

Keywords: Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8); K10/10.1; Kaposi's sarcoma (KS); Multicentric Castleman's disease (MCD); Poly(A)-binding protein (PABP); Primary effusion lymphoma (PEL)

Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV, human herpesvirus 8, HHV-8) is associated with the pathogenesis of KS, primary effusion lymphoma (PEL), and multicentric Castleman's disease (MCD) (Moore and Chang, 2001). One of the unique characteristics of this virus is that it encodes several homologues of human cytokines, cell cycle-associated genes, and chemokines. Among them, KSHV contains a cluster of interferon regulatory factor (IRF)-encoded genes in its genome. At least 7 homologues of IRFs have been identified in this cluster, i.e., K9 (viral IRF-1 or vIRF-1), K10, K10.1 (vIRF-4), K10.5 (vIRF-3 or LANA2), K10.7, K11, and vIRF2 (Afonina et al., 1998; Burysek et al., 1999; Cunningham et al., 2003; Lubyova and Pitha, 2000; Rivas et al., 2001). Some of these IRF homologues have been previously investigated, and their functions have been reported. vIRF-1 was found to have a similar function to human IRF-2 (hIRF-2) (Burysek et al., 1999; Li et al., 1998; Pozharskaya et al., 2004; Zimring et al., 1998). Like hIRF-2, vIRF-1 suppresses type I-interferon promoter activity and IFN-stimulated activation of IFN-stimulated gene (ISG) promoters (Afonina et al., 1998; Burysek et al., 1999; Zimring et al., 1998). vIRF-1 is also involved in oncogenesis by binding cellular p53 and CBP/p300 (Burysek et al., 1999). vIRF-2 suppresses interferon promoter activity and binds to IRF-1, IRF-2, ICSBP, and CBP (Burysek and Pitha, 2001; Burysek et al., 1999). vIRF-2 interacts with double-stranded RNA-activated protein kinase and inhibits antiviral effects of interferon (Burysek and Pitha, 2001). vIRF-3 (LANA2) is unique among vIRFs (Lubyova et al., 2004; Lubyova and Pitha, 2000; Rivas et al., 2001). Its kinetics are those of the latent proteins, and almost all KSHV-infected B-cells express vIRF-3 in their nucleus (Rivas et al., 2001). Moreover, vIRF-3 inhibits p53-dependent apoptosis by binding to p53 and CBP/p300, suggesting that vIRF-3 plays an important role in pathogenesis of malignancies with a latent infection with KSHV (Lubyova et al., 2004; Rivas et al., 2001). Thus, KSHV-encoded IRFs show

* Corresponding author. Fax: +81 3 5285 1189.
E-mail address: katano@nih.go.jp (H. Katano).

0042-6822/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2006.04.009

various functions, and their expression varies among genes. On the other hand, other vIRFs, e.g., K10, K11, vIRF4 (K10.1), and K10.7, have been poorly characterized, and their functions are still unknown (Cunningham et al., 2003). We previously showed that expression of K10 protein was induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in the KSHV-infected PEL cell lines, suggesting that K10 protein belonged to a family of lytic proteins (Katano et al., 2000b). Histologically, K10 protein is expressed in the nucleus by very few tumor cells in Kaposi's sarcoma (KS) tissues, although a small number of mantle zone B cells express K10 protein in their cytoplasm in multicentric Castleman's disease (MCD) tissues (Katano et al., 2000b). Furthermore, the length of ORF K10 gene is more than 2 kbp, which makes it the longest gene among all vIRFs (Cunningham et al., 2003). Transcriptional analysis revealed that ORF K10 was transcribed with ORF K10.1 as a fusion gene (K10/10.1 gene) (Cunningham et al., 2003; Jenner et al., 2001). Even though a part of the K10 gene is homologous to hIRFs, a large part of it does not correspond to any other human genes. Therefore, we hypothesized that K10/10.1 protein, a product of K10/10.1 gene, had other functions besides those of IRFs. In the present study, we revealed that the K10/10.1 transcript produced a 110-kDa protein (K10/10.1 protein), and we identified poly (A)-binding protein as a binding partner of the K10/10.1 protein.

Results

Cloning of the K10/10.1 transcript from a cDNA library

To identify transcripts encoded by the K10 gene, we immunoscreened a cDNA library. When 1×10^6 clones of a cDNA library constructed from TY-1 cells were screened using the anti-K10 polyclonal antibody (Katano et al., 2000b), 8 positive clones were obtained. Sequence analysis revealed that these clones consisted of 3 groups. The first group contained a 2921-bp cDNA with a poly(A) signal sequence corresponding to the K10/10.1 transcript (Jenner et al., 2001). The 2 other clone groups were shorter than the K10/10.1 group, and only coded for the K10 gene characterized as a 2281-bp fragment (bases 88,286–86,006 of GenBank acc. no. U75698) or a 2080-bp fragment (bases 88,085–86,006 of GenBank acc. no. U75698). To confirm expressions of these three forms of K10 transcripts, we performed Northern blot hybridization using the K10 gene as a probe. Northern blotting demonstrated that two bands were induced in TY-1 cells with TPA stimulation (Fig. 1A). The size of the longest band was 2.9 kb, possibly corresponding to the K10/10.1 transcript (Jenner et al., 2001). No band was found at 2281 and 2080 b. In addition to the band of the K10/10.1 transcript, a strong 1.0-kb band was observed in stimulated PEL cells, suggesting the presence of another form of the K10 gene. Unstimulated PEL cells demonstrated two

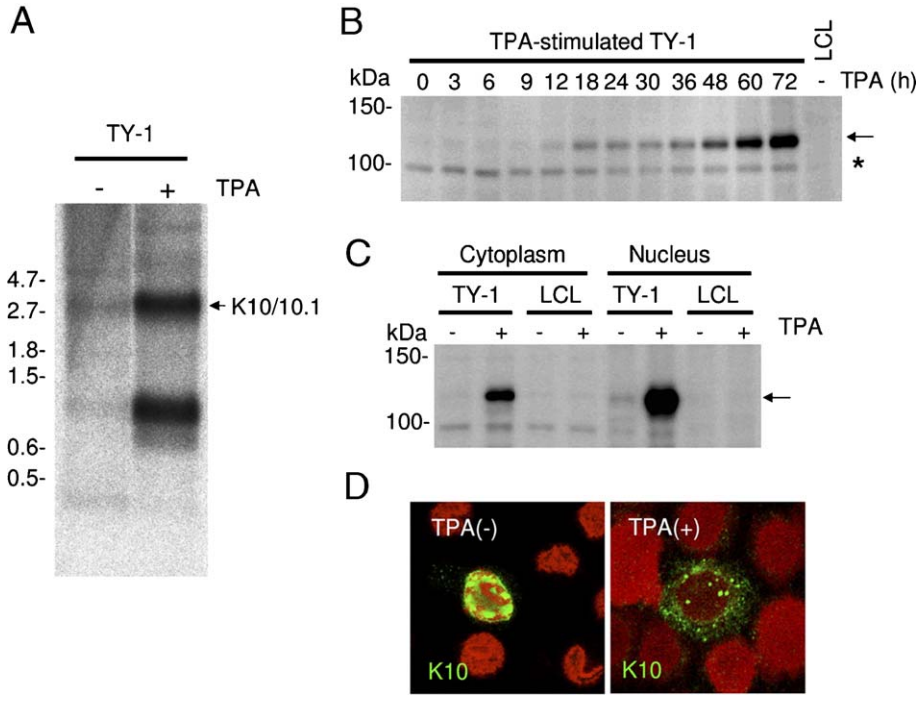

Fig. 1. Expression of K10/10.1 gene and protein in a PEL cell line. (A) Northern blot hybridization. mRNAs extracted from TPA-stimulated TY-1 (+) or unstimulated TY-1 (−) cells were electrophoresed and blotted on a membrane. Radiolabeled K10 DNA (amplified by PCR) was used as probe. The arrow indicates the K10/10.1 gene transcript. The lower band at 1.0 kb represents another transcript of the K10 gene. (B) Western blot analysis for K10/10.1 protein in TPA-stimulated TY-1 cells. Numbers of hours after addition of TPA are shown at the top of the panel. LCL: lymphoblastoid cell line as a negative control. The arrow indicates specific bands obtained with the anti-K10 rabbit polyclonal antibody, while lower bands (asterisk) are non-specific. (C) Western blot analysis for K10/10.1 protein in subcellular fractions. The arrow indicates K10/10.1 protein-specific bands. (D) IFA of K10/10.1 protein in unstimulated (left) and TPA-stimulated TY-1 (right) cells using anti-K10 rabbit polyclonal antibody. K10/10.1 protein is represented in green color. The red color indicates nuclear counterstaining with propidium iodide.

weak bands at 2.9 kb and 1.0 kb. Thus, we concluded that a 2.9-kb transcript of K10/10.1 was the major transcript of the K10/10.1 gene, and that 2 other short clone groups were artificially present.

Expression and kinetics of K10/10.1 gene/protein in PEL cell lines

Several studies reported kinetics of KSHV-encoded genes in PEL cell lines (Fakhari and Dittmer, 2002; Jenner et al., 2001; Sun et al., 1999). We previously demonstrated that K10 protein was induced by TPA, suggesting that K10 protein was a lytic protein (Katano et al., 2000b). DNA array analysis by another group suggested the presence of 2 transcripts including the K10 gene, i.e., K10/10.1 and K10 (Jenner et al., 2001). Cluster analysis and RT-PCR suggested that K10 might be a latent gene, whereas K10/10.1 was a lytic gene (Jenner et al., 2001). Thus, we examined expressions of K10/10.1 gene and protein in PEL cell lines. Northern blot analysis demonstrated that K10/10.1 transcript (2.9 kb) was induced by TPA in TY-1 cells (Fig. 1A). Western blotting demonstrated that an anti-K10 antibody reacted with a 110-kDa protein of K10/10.1, and that amount of K10/10.1 protein increased after addition of TPA (Fig. 1B). Subcellular fractionation revealed that K10/10.1 protein was expressed mainly in the nucleus, and partly in the cytoplasm (Fig. 1C). IFA using anti-K10 antibody showed that K10/10.1 protein was expressed in the nucleus of a very small population of unstimulated PEL cells (Fig. 1D). When stimulated with TPA, the number of K10/10.1-positive cells increased. Dot-like signals were observed not only in the nucleus, but also in the cytoplasm of TPA-stimulated TY-1 cells (Fig. 1D). In addition, most of K10/10.1-positive cells expressed K10/10.1 protein in the nucleus, while some cells expressed K10/10.1 both in the cytoplasm and the nucleus. We also examined another KSHV-infected PEL cell line, BCBL-1, and obtained similar results (data not shown).

Expression of K10/10.1 protein in KS, MCD, and KSHV-associated solid lymphoma

We previously developed a polyclonal antibody to K10 protein, and reported expression of K10 protein in KS and MCD tissues (Katano et al., 2000b). As we showed in Fig. 1, Western blot analysis revealed that this anti-K10 antibody reacted with K10/10.1 protein predominantly (Fig. 1). In order to further reveal the detailed expression of K10/10.1 protein in KSHV-associated diseases, we performed immunohistochemistry for K10/10.1 protein in additional cases of KS, MCD, and in an animal model of KSHV-associated solid lymphoma (Katano et al., 2000b). As it was previously reported (Katano et al., 2000b),

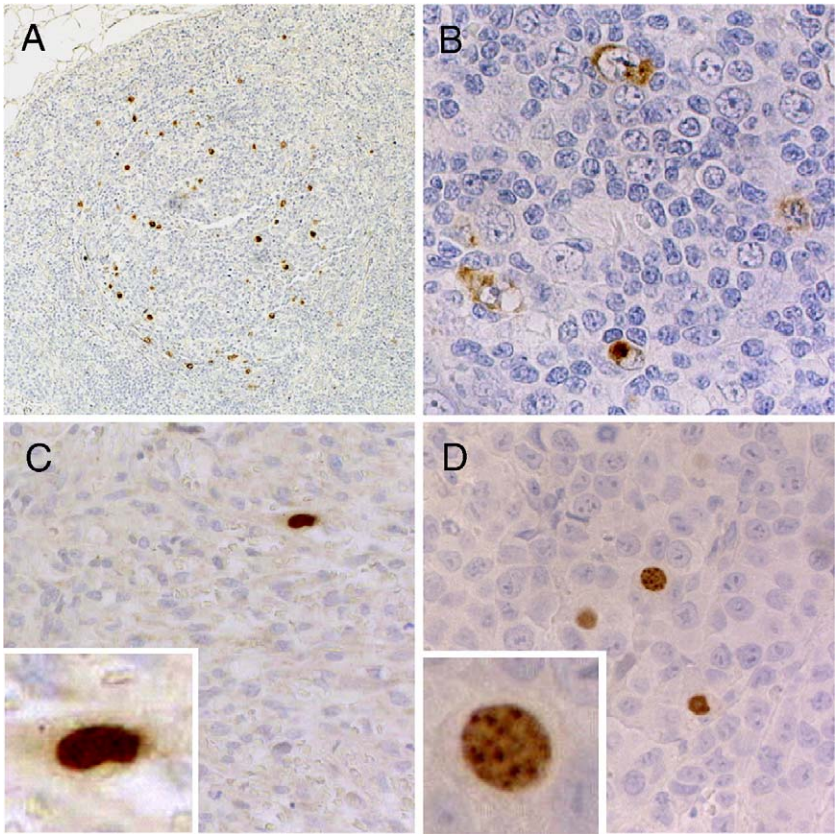

Fig. 2. Immunohistochemistry of K10/10.1 protein in KSHV-associated diseases. (A and B) Lymph node from a patient with MCD. The low power view shows K10/10.1 protein-positive cells in the mantle zone of the germinal center in a MCD lesion (A). Some B cells in the mantle zone show positive signals in the cytoplasm (B). (C) Kaposi's sarcoma (KS). One cell expresses K10/10.1 protein in this panel. In the high power view, diffuse staining is present in the nucleus (inset). (D) An animal model of KSHV-associated solid lymphoma. Some of the lymphoma cells are positive for K10/10.1 protein. In the high power view, the signal is seen as a dot-like staining pattern in the nucleus (inset).

we found that K10/10.1 protein was expressed predominantly in the cytoplasm of B cells in the mantle zone of MCD lesions (Figs. 2A and B). Expression of K10/10.1 protein was observed in the nucleus of KS cells, and the frequency of K10/10.1-positive cells was very low (less than 1%) in KS lesions (Fig. 2C). In an animal model of KSHV-associated solid lymphoma, K10/10.1 protein was expressed in the nucleus of lymphoma cells (Fig. 2D). A careful observation revealed that staining with the anti K10 antibody showed a dot-like pattern in the nucleus (Fig. 2D, inset).

Localization of K10/10.1 in transfectants

To investigate function and expression of K10/10.1 protein, we constructed a plasmid expressing K10/10.1 protein. A transfection study demonstrated that the expression plasmid produced a 110-kDa protein that reacted with anti-K10 antibody in 293T cells (Fig. 3A). IFA revealed its subcellular localization in K10/10.1-transfected HeLa cells (Fig. 3B). While K10/10.1 protein was expressed predominantly in the nucleus in 30% of transfectants, 60% of transfected cells expressed K10/10.1 protein in both the cytoplasm and nucleus, and the remaining 10% of cells showed K10/10.1 protein predominantly in the cytoplasm. We next constructed GFP-tagged plasmids expressing the K10/10.1 protein, and transfected them into HeLa cells (Fig. 3C). Similar localization was observed in the GFP-K10/10.1-transfected HeLa cells. Although PROSITE, an online protein domain searchable database (http://us.expasy.org/prosite/), did not detect any putative sequence of nuclear localization signals in the K10/10.1 gene, our data suggested that K10/10.1 might have unknown nuclear localization signals or DNA binding domains.

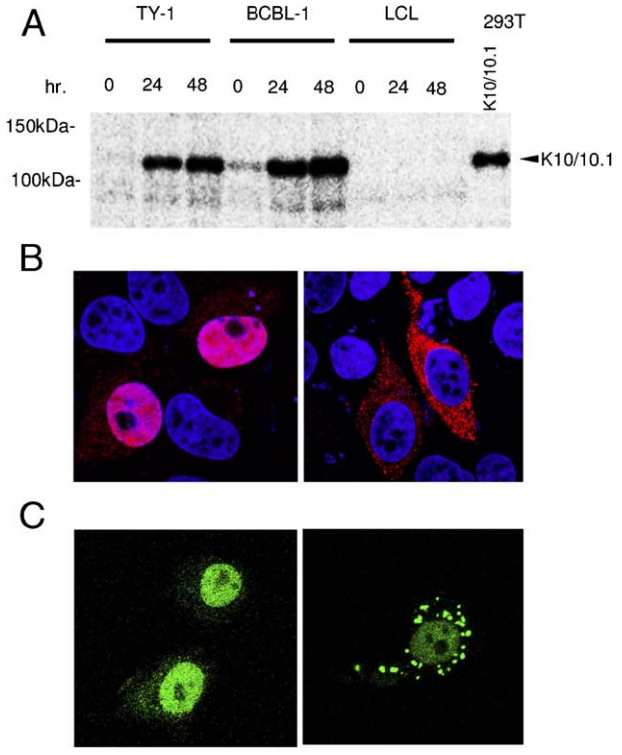

Fig. 3. Expression of K10/10.1 transcript in transfectants. (A) Western blot analysis. In 293T transfectants, the K10/10.1 transcript produced a protein with a similar size to that of K10/10.1 proteins in KSHV-infected PEL cell lines. (B) Localization of each form of K10 proteins in HeLa cells. The red color indicates K10/10.1 protein, and the blue color is the nucleus (TOPRO3). K10/10.1 protein is present predominantly in the nucleus (left panel) and rarely in the cytoplasm (right panel). (C) Localization of GFP-K10/10.1 protein in HeLa cells. GFP signal is observed predominantly in the nucleus (left panel), sometimes in both the cytoplasm and nucleus (right panel).

Functions as a homologue of IRFs

Since the K10/10.1 transcript is encoded in the cluster of vIRFs in KSHV genome, we compared the K10/10.1 protein with IRFs using multiple sequence alignments with the Clustal X software. Multiple sequence alignments demonstrated that K10.1 (the N-terminal region of the K10/10.1 protein) coded homologous regions to the DNA binding domain of IRFs, including a tryptophan pentad repeat (Fig. 4A). The N-terminus of the K10 protein, which localized in a middle part of K10/10.1 protein, also showed homologous regions to the DNA binding domain of IRFs. Moreover, K10/10.1 protein has some homologous regions in its C-terminus. A phylogenic tree analysis revealed that both K10 and K10.1 belonged to the same cluster as vIRF-1, suggesting that K10/10.1 protein might have similar functions to IRFs (Fig. 4B).

IFA demonstrated a dot-like staining pattern in the nucleus of PEL cell lines (Fig. 1D). It is known that vIRF-1 localizes in the promyelocytic leukemia protein (PML) bodies in the nucleus (Pozharskaya et al., 2004). Since K10/10.1 protein has a homology to vIRF-1, we investigated whether K10/10.1 protein co-localized with the PML bodies. IFA demonstrated that staining of K10/10.1 protein only partially overlapped with that of PML (Fig. 4C). Some hIRFs co-localize with the SC35 domain, a component in the nucleus close to the PML bodies (Maul, 1998). Therefore, we investigated if K10/10.1 protein co-localized with SC35. IFA revealed that signals of K10/10.1 protein overlapped with those of SC35 (Fig. 4D), suggesting that K10/10.1 protein was expressed in the SC35 domain near the PML body in the nucleus.

We then examined if K10/10.1 protein suppressed promoter activity of interferons. A reporter gene assay using pGL3-IFNB-Luc demonstrated that K10/10.1 protein did not suppress promoter activity of IFNB induced by a Sendai virus infection (Fig. 4E). These data suggested that K10/10.1 protein might not suppress promoter activity of IFNs, while K10/10.1 protein shows some homology with IRFs.

PABP binds to K10/10.1 protein

To further clarify the function of K10/10.1 protein, we next investigated its binding protein. GST-K10 fusion protein was mixed with TY-1 cell lysate, and a GST pull down assay was performed. SDS-PAGE of the pulled down proteins demonstrated the presence of a protein that specifically bound to K10 protein but not to GST in the TY-1 lysate (Fig. 5A). We excised the appropriate band from the gel, and analyzed it using matrix-

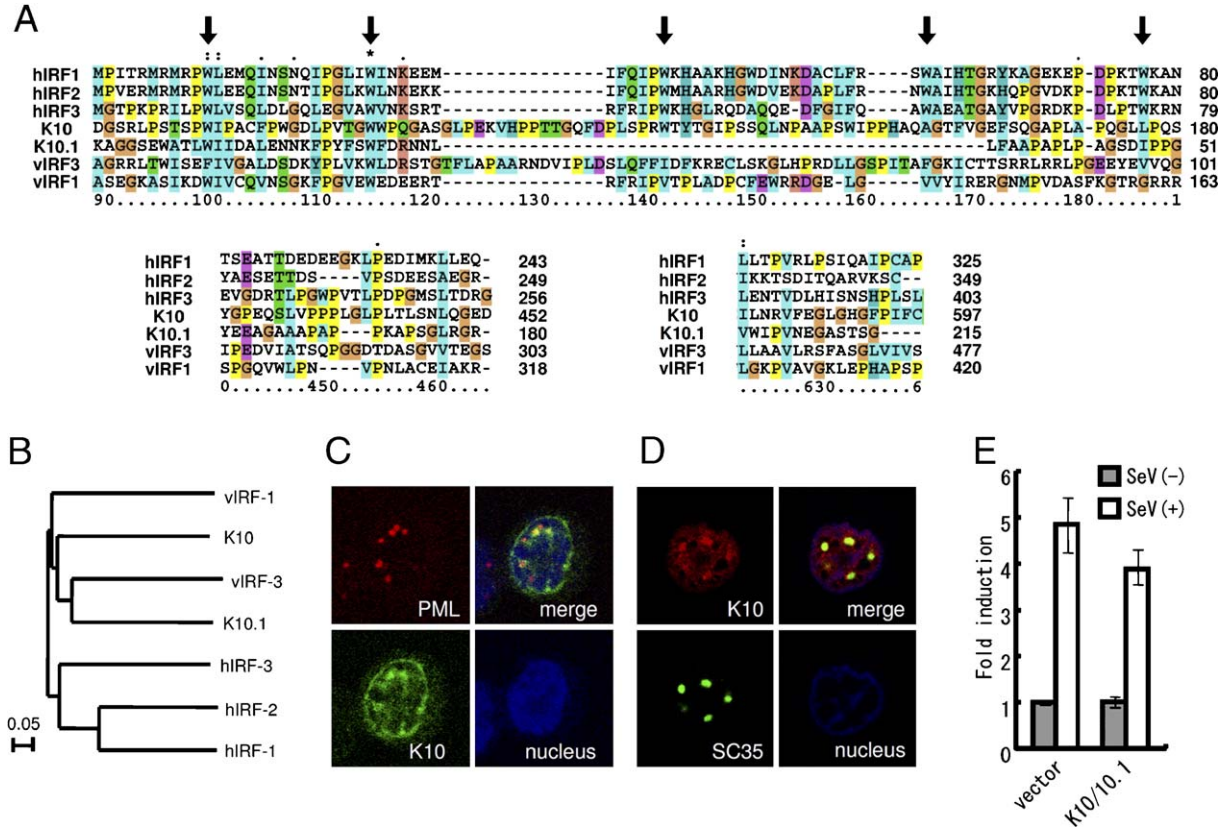

Fig. 4. K10/10.1 protein shows homologous domains to human and viral IRFs, but does not inhibit interferon promoter. (A) Alignment with IRFs using the Clustal X software. hIRF-1 (GenBank accession no. 87992), hIRF-2 (539621), hIRF-3 (4504725), vIRF-3 (AY008303), K10.1 (from 88410 to 88910 of KSU75698), K10 (from 86074 to 88164 of KSU75698), and vIRF-1 (4929348) were aligned. Arrows indicate tryptophan pentad repeats of the DNA binding domain. Numbers in the right of each panel indicate amino acids of each protein. Numbers counted automatically by the Cluster X were shown in the bottom of each panel. (B) Phylogenic tree analysis of IRFs, K10 and K10.1 proteins. The length of each branch indicates genetic distance with a scale size of 0.05 (5%). (C) Immunofluorescence of K10/10.1 protein and PML in TPA-stimulated TY-1 cells. PML and K10/10.1 protein were stained with Alexa 568 (red, upper left panel) and Alexa 488 (green, lower left panel), respectively. The nucleus was counterstained with TOPRO3 (blue, lower right panel). In the merged image (upper right), overlapping of the three colors is shown in white color. (D) Immunofluorescence of K10/10.1 protein and SC35 in TPA-stimulated TY-1 cells. (E) Reporter gene assay of IFNB promoter. The pGL3-IFNB-Luc reporter plasmid was co-transfected into HeLa cells with pBK-CMV vector (vector) and K10/10.1 expression plasmids. Cells were infected with Sendai virus 24 h after transfection, for 16 h, and analyzed for Luc activity. Error bars show standard deviation for triplicate experiments.

assisted laser desorption ionization/time-of-flight (MALDI-ToF) mass analysis. Mass spectrometry revealed that the protein corresponded to human poly(A)-binding protein (PABP), cytoplasmic 1. To confirm the binding of PABP to GST-K10 protein, we performed immunoblot analysis of the lysate obtained in the GST pull down (Fig. 5B). PABP was detected in the lysate pulled down with GST-K10 protein in the TY-1 lysate. In addition, Western blotting demonstrated that PABP was detected in the lysate immunoprecipitated with anti-K10 antibody in TY-1 and BCBL-1 cells (Fig. 5C). Moreover, K10/10.1 protein was detected in the lysate immunoprecipitated with anti-PABP antibody in cells (Fig. 5D). These data suggested that PABP bound to K10/10.1 protein in KSHV-infected PEL cells. To identify the binding site of PABP in K10/10.1 protein, we constructed plasmids expressing various deletion mutants of K10/10.1 protein (Fig. 6A). Immunoprecipitation clearly demonstrated that PABP bound to a 157–380 amino acid region of K10/10.1 protein (Fig. 6B). Finally, we investigated co-localization of PABP and K10/10.1 protein in KSHV-infected cells. In KSHV-infected PEL cells, TPA-stimulation induced K10/10.1 expression in the nucleus and cytoplasm (Fig. 1D). Interestingly, IFA revealed that PABP co-localized with K10/10.1 protein predominantly in the nucleus, but not in the cytoplasm (Fig. 7). Furthermore, in the nucleus of some cells, both K10/10.1 protein and PABP showed dot-like staining patterns, and co-localized in these dots. Since PABP localizes in the SC35 domain close to the PML bodies, and binds to poly(A) RNA in the nucleus (Afonina et al., 1998), these data suggested that K10/10.1 protein and PABP co-localized in the SC35 domain of TY-1 cells, and K10/10.1 protein might play a role in the function of PABP in the nucleus of KSHV-infected cells.

Discussion

In the present study, we characterized KSHV-encoded K10/10.1 protein. Our results showed that K10/10.1 protein was rarely expressed in the nucleus of KS cells, but was frequently found in the cytoplasm of the mantle zone B cells in MCD lesions. While functions of K10/10.1 protein are still unclear, K10/10.1 protein was identified as the longest homologue to

vIRFs, and its gene contained long sequences with no homology to IRFs. Thus, it is possible that K10/10.1 protein may have different functions from those of other vIRFs or hIRFs. Specifically, we showed that K10/10.1 protein did not influence promoter activity of interferons, and binding of K10/10.1 protein to PABP suggested that K10/10.1 protein affected host translation. Thus, K10/10.1 protein may play a different role from those of other viral IRFs in KSHV pathogenesis, while the K10/10.1 gene is encoded in the cluster of viral IRFs.

Although KSHV has been detected in KS, lymphoma, and MCD, association with KSHV differs among these KSHV-associated diseases (Katano et al., 2000b; Moore and Chang, 2001). We previously revealed that almost all KS cells expressed only latent gene products, and expression of lytic proteins was rare (Katano et al., 2000b). On the other hand, various lytic proteins encoded by KSHV, e.g., vIL-6, ORF59, and K8, have been detected in MCD lesions. These observations strongly suggested that lytic replication of KSHV was crucial in pathogenesis of MCD, whereas latency of KSHV was crucial in KS cells. Results of our immunohistochemical experiments demonstrated different subcellular localizations of K10/10.1 protein between KS and MCD. This differential subcellular localization of K10/10.1 protein was also observed between unstimulated and TPA-stimulated KSHV-infected PEL cell lines. Cytoplasmic localization of K10/10.1 protein was observed only in TPA-stimulated cells. Therefore, cytoplasmic staining of K10/10.1 protein in MCD lesions suggested that B cells in the mantle zone would be in the lytic phase of KSHV infection in MCD. Other KSHV-encoded proteins such as ORF59 demonstrated different subcellular localizations between unstimulated and TPA-stimulated TY-1 or BCBL-1 as well as between KS and MCD (unpublished data). However, expression of K10/10.1 protein demonstrated a very clear difference in MCD compared with that of ORF59. Therefore, it is possible that an anti-K10 antibody will provide a useful tool for the diagnosis of KSHV-associated MCD.

PABP is a multifunctional protein and is an important molecule for mRNA stabilization, translation initiation, protection of poly(A) from nuclease activity, mRNA deadenylation, and mRNP maturation in host cells (Grosset et al., 2000; Guhaniyogi and Brewer, 2001; Minvielle-Sebastia et al., 1997). In particular, PABP may play an important role in regulating mRNA turnover by inhibiting mRNA decapping with its poly (A)-tail (Khanna and Kiledjian, 2004). PABP is predominantly present in the cytoplasm, although it is also found in the nucleus (Afonina et al., 1998). A study using GFP-tagged PABP revealed that PABP shuttled between the cytoplasm and the nucleus (Afonina et al., 1998). In the nucleus, PABP predominantly co-localizes with SC35, a splicing factor, and interacts with the poly(A)-tail (Afonina et al., 1998). It is also known that SC35 is localized close to the PML bodies in the nucleus (Maul, 1998). In the present study, immunoprecipitation suggested that a limited amount of K10/10.1 protein actually bound to PABP in KSHV-infected cells (Figs. 5C and D). IFA

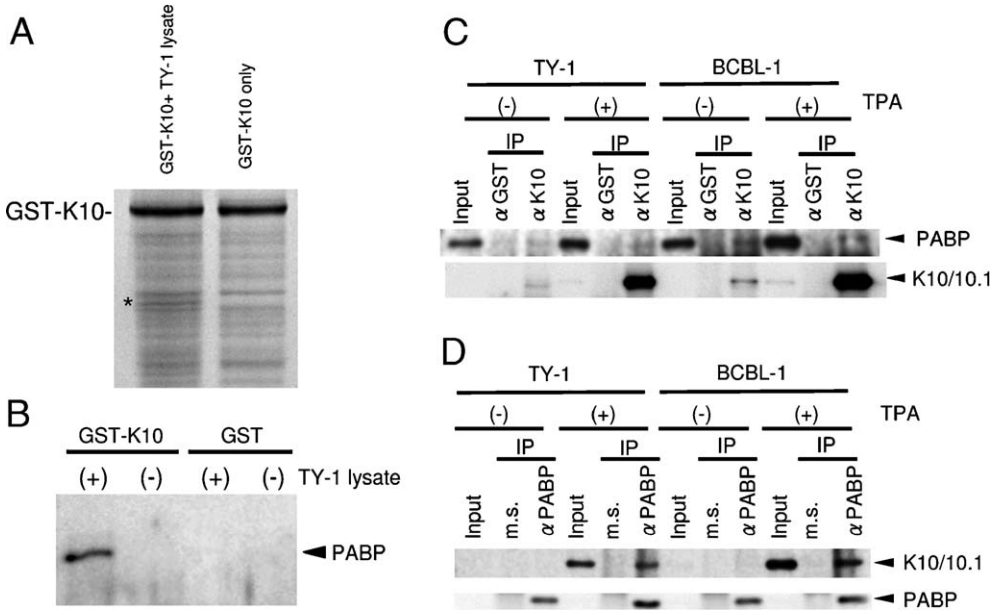

Fig. 5. K10/10.1 protein binds to PABP. (A) GST pull down assay. Ten micrograms of GST-K10 protein was incubated with TY-1 cell lysate. After adding glutathione–Sepharose beads, the beads were washed 3 times with lysis buffer. Then, the beads were loaded for SDS-PAGE using 2× sample buffer. The gel was stained with Coomassie brilliant blue. The asterisk indicates a band observed only in the lane of GST-K10 protein + TY-1 lysate, and not in the GST-K10 protein lane; as well as not in the GST protein + TY-1 lysate lane and the GST protein only lane (data not shown). (B) Western blotting of GST pulled down lysates. Beads obtained from the GST pull down assay were electrophoresed using SDS-PAGE, and blotted on a membrane. The membrane was stained with anti-PABP antibody. (C) Immunoprecipitation (IP) with anti-K10 antibody and immunoblotting with PABP. Lysates from TY-1 and BCBL-1 were immunoprecipitated with anti-K10 antibody or anti-GST antibody. Recombinant protein A-Sepharose was added and incubated. Sepharose was electrophoresed and blotted. Anti-PABP antibody was used as primary antibody for immunoblotting. Input indicates the whole lysate of TY-1 or BCBL-1 without IP. In the lower panel, anti-K10 antibody was used as primary antibody on the same membrane after stripping. (D) Immunoprecipitation with anti-PABP antibody and immunoblotting with anti-K10 antibody. Lysates from TY-1 and BCBL-1 were immunoprecipitated with anti-PABP antibody or normal mouse serum (m.s.). In the immunoblot, anti-K10 antibody was used as primary antibody for immunoblotting. In the lower panel, anti-PABP antibody was used as primary antibody on the same membrane after stripping.

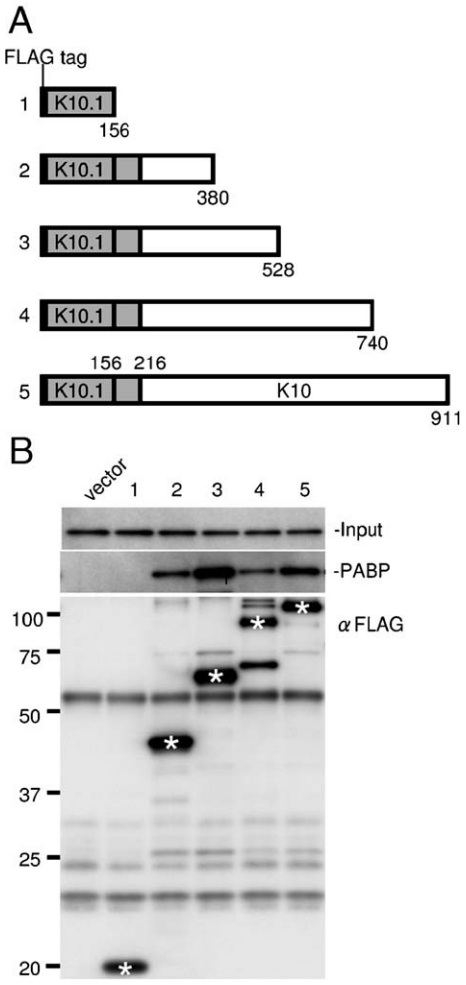

Fig. 6. Identification of the binding site of K10/10.1 protein with PABP. (A) Constructs of FLAG-tagged deletion mutants. K10/10.1 protein is composed of 911 amino acids. Four deletion mutants and a full-length K10/10.1 protein were constructed. (B) Immunoprecipitation. K10/10.1 mutants were immunoprecipitated with anti-FLAG antibody and were separated by SDS-PAGE. Endogenous PABP was detected in the top panel. Co-precipitated PABP with mutants are shown in the middle panel. Numbers on the top of the panel correspond to those in panel A. The lower panel shows sizes of deletion mutants by immunoblot using anti-FLAG antibody. Each band of mutant is indicated by white asterisks.

showed that K10/10.1 protein co-localized with PABP only in the nucleus (Fig. 7), whereas both PABP and K10/10.1 protein were present in the nucleus and cytoplasm. This might be one of the reasons why a limited amount of K10/10.1 protein bound to PABP. Overall, we can conclude that PABP forms a large complex near the PML bodies in the nucleus with SC35 and K10/10.1 protein, and is associated with mRNA turnover. It is known that some viral proteins such as poliovirus 3C protease and enterovirus proteases bind to, and cleave PABP, shutting off host translation (Joachims et al., 1999; Kuyumcu-Martinez et al., 2004). We investigated the amount of PABP in K10/10.1-transfected cells, but failed to detect changes in PABP expression levels (Kanno et al., unpublished data). Thus, K10/10.1 protein did not, at least in transfected cells, seem to directly alter PABP amount. However, our results suggested that K10/10.1 protein might inhibit function of PABP predominantly in the nucleus, resulting in shutoff of host translation. In KS cells, chemotherapy regimens including doxorubicin, bleomycin, and vincristine dramatically reduce amounts of cytoplasmic PABP, resulting in translation shutoff and G2 arrests in host cells (van der Kuyl et al., 2002). Further studies are required to clarify relationships between K10/10.1 protein and PABP.

Materials and methods

Cell culture

KSHV-harboring TY-1 cells (Katano et al., 1999b) and BCBL-1 cells (AIDS Research and Reference Reagent Program #3233, National Institutes of Health, Bethesda, MD) were cultured in RPMI 1640, in the presence of 10% FCS. HeLa and 293T cells were cultured in DMEM with 10% FCS.

Immunoscreening

A cDNA library was constructed from TPA-stimulated TY-1 cells and a KSHV-infected cell line, using *Eco*RI-predigested lambda ZAP Express vectors according to the manufacturer's instructions (Stratagene, La Jolla, CA). Immunoscreening was performed as described previously (Katano et al., 1999a). Approximately 1×10^6 phages were screened on nitrocellulose filters using an anti-K10 polyclonal antibody (Katano et al., 2000b). The antibody was diluted 1:2000 in 1 × Block Ace (Snow Brand Milk Products, Tokyo, Japan), and incubated with filters for 1 h at room temperature. Filters were washed in phosphate-buffered saline (PBS)-0.1% Tween 20, reacted with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG, 1:5,000; Biosource), and visualized with nitroblue tetrazolium (NBT) and 5-bromo-1-chloro-3-indolylphosphate (BCIP, Promega, Madison, WI). Positive phages were selected, screened twice by the same protocol, and examined for their reactivity with the anti-K10 antibody. After conversion of positive phages into phagemids using the rapid excision system (Stratagene) and helper phages according to the manufacturer's instructions, inserts were sequenced with an ABI Prism 310 sequencer (Applied Biosystems, Foster City, CA) with the use of internal sequencing primers.

Northern blotting

Messenger RNA was extracted from TY-1 and BCBL-1 cells using a Fast Track 2.0 mRNA isolation kit (Invitrogen, Carlsbad, CA). Fifteen micrograms of mRNA were separated on 1% formaldehyde-containing agarose gel, transferred to a nylon filter, and hybridized with a probe. K10 PCR products (forward primer, 5′-CTCGGATCCCATCTACGTCCCCGTGGATA-3′; reverse primer, 5′-CTCGAATTCTGTAGATGCCGGGGATGCGC-3′; template DNA, TY-1 DNA; product size, 1767 bp) were labeled with ^{32}P (High Prime, Roche Applied Science, Mannheim, Germany), and used as probe.

Western blotting

Cell extract preparation and immunoblots were performed as described previously (Katano et al., 2001). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), probed with anti-K10 rabbit polyclonal antibody (Katano et al., 2000b) or anti-PABP mouse monoclonal antibody (ImmuQuest, Santa Cruz, CA), followed by incubation with horseradish peroxidase-conjugated anti-rabbit (or mouse) antibodies (Biosource International, Camarillo, CA), and visualization using ECL plus (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Subcellular fractionation

Subcellular fractionation was prepared for TY-1 and LCL cells with/without TPA stimulation. 1×10^7 cells were lyzed in hypotonic buffer (20 mM HEPES pH 7.0, 10 mM KCl, 1 mM $MgCl_2$, 0.5 mM DTT, 0.1% Triton X-100) for 20 min on ice. After voltexing for 30 s, lysates were centrifuged at 13,000×*g* for 10 s. Supernatants were used as cytoplasmic fractions. Pellets were washed with hypotonic buffer twice, lyzed in extraction buffer (20% Glycerol, 420 mM NaCl, 20 mM HEPES pH 7.0, 10 mM KCl, 1 mM $MgCl_2$, 0.5 mM DTT, 0.1% Triton X-100), and centrifuged. Supernatants were used as nuclear extracts. A protease-inhibitor cocktail (Complete, EDTA-free, Roche Diagnostics, Indianapolis, IN) was added to the buffer before use.

Immunofluorescence assay (IFA)

IFA was performed as described previously (Katano et al., 2001). Anti-K10 (Katano et al., 2000b), PML (Santa Cruz Biotechnology, Santa Cruz, CA), SC35 (Sigma-Aldrich, St. Louis, MO), or PABP (ImmuQuest) antibodies were used as primary antibodies. Secondary antibodies were either Alexa-488 or Alexa-568 conjugated anti-rabbit (or anti-mouse) IgG antibodies (Molecular Probe, Eugene, OR). TOPRO3 (Molecular Probe) was used for counterstaining cell nuclei. Imaging was performed using a confocal microscope equipped with an argon–krypton laser (LSM-MicroSystem, Zeiss, Germany).

Immunohistochemistry

Formalin-fixed specimens of KS, MCD, and the animal model of KSHV-associated solid lymphoma were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Immunohistochemistry was performed with an anti-K10 rabbit polyclonal antibody (Katano et al., 2000b). For second and third phase reagents of immunostaining, a biotinylated F(ab′)2 fragment of goat anti-rabbit immunoglobulin (DAKO, Copenhagen, Denmark) and peroxidase-conjugated streptavidin (DAKO) were used. Details regarding immunostaining methods have been described previously (Katano et al., 2000b). An animal model of KSHV-associated solid lymphoma was established as described previously (Katano et al., 2000c). 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., 2000c).

Plasmids

Expression vectors of K10/10.1 were obtained using an in vivo excision system from phage plaques (Stratagene). To construct pEGFP-K10/10.1, the K10/10.1 gene was amplified by PCR using forward (5′-CTACTCGAGCCTAAAGCCGGTGGCTCAGAATGG-3′) and reverse (5′-CGGGGATCCTCAATGTAGACTATCCCAAATGGA-3′) primers from the expression plasmid. PCR products were cloned into the *Xho*I/*Bam*HI sites of pEGFP (BD Biosiences Clontech, Mountain

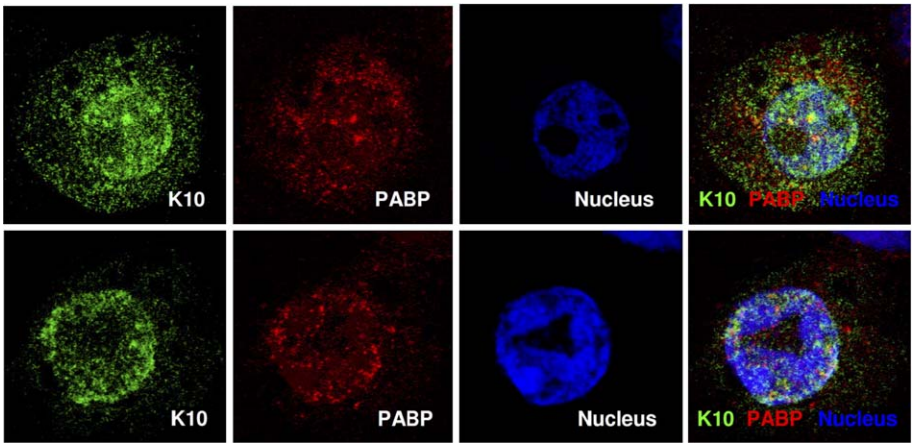

Fig. 7. IFA of TPA-stimulated TY-1 cells. K10/10.1 protein and PABP co-localize in the nucleus, but not in the cytoplasm. In some cells, K10/10.1 protein is present in the nucleus as a patchy pattern, and is diffusely distributed in the cytoplasm (upper panels). PABP co-localizes with K10/10.1 protein in the nucleus, as a patchy staining pattern in the upper panels. In the lower panels, K10/10.1 protein is expressed predominantly in the nucleus and co-localizes with PABP as a dot-like staining pattern near the nuclear membrane in the nucleus.

View, CA). For the reporter assay of interferon regulatory factors, pIFNB-Luc was constructed as reported previously (Lubyova et al., 2004). For constructions of deletion mutants, 5 fragments of K10/10.1 were amplified with PCR using one forward primer (5′-CTAGAATTCCCTAAAGCCGGTGGCTCAGAATGG-3′) and 5 reverse primers (5′-TAAGCGGCCGCTCAAACCTCACACCCCCTTC-3′ for amino acids no. 1–156, 5′-TAAGCGGCCGCTTAGAACTCACCGACAAATGTTCCCGC-3′ for amino acids no. 1–380, 5′-TAAGCGGCCGCTGGCTCCTGCGCTCTGCGACCTCT-3′ for amino acids no. 1–528, 5′-CGAGCGGCCGCTCAAAAGATTTCCACAACAAAAGACAC -3′ for amino acids no. 1–740, 5′-CATGCGGCCGCTCAATGTAGACTATCCCAAATGGA-3′ for full length of K10/10.1). PCR products were cloned into the *Eco*RI site of pME18FLAG (Ishida et al., 1996).

Transfection and luciferase assays

Expression plasmids were transfected into HeLa or 293T cells using Lipofectamine Plus (Invitrogen) for HeLa cells or Fugene 6 (Roche Diagnostics, Indianapolis, IN) for 293T cells, according to the manufacturer's instructions. An IFN promoter assay was performed as reported previously (Lubyova et al., 2004). Briefly, K10/10.1 expression plasmid and empty vector were co-transfected with pGL3-IFNB-Luc and phRL-CMV vector into HeLa cells with lipofectamine Plus (Invitrogen). Twenty-four hours after transfection, wells were washed, and Sendai virus was added to 1% BSA phosphate-buffered saline with a m.o.i of 5. Luciferase activity was measured using a dual luciferase assay system (Promega).

GST pull down assay

To identify the binding protein to K10/10.1 protein, a glutathione *S*-transferase (GST) pull down assay was performed. GST-K10 fusion gene was generated by ligation of a *Bam*HI–*Eco*RI-digested PCR product of the K10 gene to the *Bam*HI–*Eco*RI site of *p*GEX 5X-2 (Amersham Pharmacia Biotech) (Katano et al., 2000a). GST-K10 fusion protein was then expressed in *Escherichia coli*, and affinity-purified using glutathione–Sepharose as described previously (Katano et al., 2000a). Purity and concentration of eluted proteins were determined by SDS-PAGE and the Bradford assay (Protein Assay; BioRad, New York, NY), respectively. TY-1 cells (1×10^7) were washed with PBS and lyzed in 1 ml lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 100 U/ml aprotinin, and 0.02% sodium azide). After absorption of glutathione–Sepharose beads (Amersham Pharmacia Biotech), 10 μg GST-K10 protein or GST protein that was purified with a glutathione–Sepharose 4B column was added, and incubated at 4 °C for 2 h. Then, 25 μl glutathione–Sepharose beads were added, and incubated for 1 h. The beads were washed 3 times in carbonate buffer containing 100 mM $NaHCO_3$ and 300 mM NaCl. Bound proteins were eluted with an equal volume of 2× sampling buffer and separated by SDS-PAGE.

Mass spectrometry

Bands of interest were excised from Coomassie-stained gels, followed by destaining, reduction, alkylation, and digestion with modified porcine trypsin (Promega). Samples for MALDI-ToF analysis were prepared by mixing a small aliquot of the digested supernatant with an equal volume of a solution of alpha-cyano-4-hydroxycinnamic acid (10 mg/ml in 1:1 acetonitrile: 0.1% vol/vol trifluoroacetic acid). Peptide mass fingerprinting was performed on a reflectron MALDI-ToF mass spectrometer (Voyger, Amersham Biosciences). The Protein Prospector (http://prospector.ucsf.edu/) was employed for protein database search using monoisotopic mass values for each spectrum.

Immunoprecipitation

Cells (1×10^6) were lyzed in 1 ml lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 100 U/ml aprotinin, 1 mM DTT). After absorption of recombinant protein A–Sepharose (Amersham Pharmacia Biotech, Buckinghamshire, UK), equal amounts of cell lysates were incubated with equal amounts of a polyclonal antibody against the K10 protein or preimmune-rabbit serum. The proteins separated by SDS-PAGE were transferred onto membranes (Immobilon; Millipore, Bedford, MA).

Deletion mutants

Plasmids expressing mutant K10/10.1 were transfected in 293T cells. Transfectants were lyzed, and equal amounts of lysate were incubated with anti-FLAG M2 antibody (Sigma). Recombinant protein A-Sepharose was added, and proteins were separated by SDS-PAGE. Endogenous PABP was detected on a blotted membrane using anti-PABP antibody.

Acknowledgments

The authors would like to thank Dr. Atsushi Kato of the Department of Virology 3 at the National Institute of Infectious Diseases for providing Sendai virus samples, and Drs. Fumiko Shinkai-Ouchi and Yoshio Yamakawa of the Department of Biochemistry and Cell Biology at the National Institute of Infectious Diseases for their technical assistance on mass spectrometry. This research was supported by Health and Labor Sciences Research Grants on HIV/AIDS and Measures for Intractable Diseases from the Ministry of Health, Labor and Welfare (grants H15-AIDS-005 to H.K., and 17243601 to T.S.), a Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (grant 17590365 to H.K.), and a grant for Research on Health Sciences focusing on Drug Innovation from Japan Health Sciences Foundation (grant SA14831 to H.K).

References

Afonina, E., Stauber, R., Pavlakis, G.N., 1998. The human poly(A)-binding protein 1 shuttles between the nucleus and the cytoplasm. J. Biol. Chem. 273, 13015–13021.

Burysek, L., Pitha, P.M., 2001. Latently expressed human herpesvirus 8-encoded interferon regulatory factor 2 inhibits double-stranded RNA-activated protein kinase. J. Virol. 75, 2345–2352.

Burysek, L., Yeow, W.S., Lubyova, B., Kellum, M., Schafer, S.L., Huang, Y.Q., Pitha, P.M., 1999. Functional analysis of human herpesvirus 8-encoded viral interferon regulatory factor 1 and its association with cellular interferon regulatory factors and p300. J. Virol. 73, 7334–7342.

Cunningham, C., Barnard, S., Blackbourn, D.J., Davison, A.J., 2003. Transcription mapping of human herpesvirus 8 genes encoding viral interferon regulatory factors. J. Gen. Virol. 84, 1471–1483.

Fakhari, F.D., Dittmer, D.P., 2002. Charting latency transcripts in Kaposi's sarcoma-associated herpesvirus by whole-genome real-time quantitative PCR. J. Virol. 76, 6213–6223.

Grosset, C., Chen, C.Y., Xu, N., Sonenberg, N., Jacquemin-Sablon, H., Shyu, A.B., 2000. A mechanism for translationally coupled mRNA turnover: interaction between the poly(A) tail and a c-*fos* RNA coding determinant via a protein complex. Cell 103, 29–40.

Guhaniyogi, J., Brewer, G., 2001. Regulation of mRNA stability in mammalian cells. Gene 265, 11–23.

Ishida, T.K., Tojo, T., Aoki, T., Kobayashi, N., Ohishi, T., Watanabe, T., Yamamoto, T., Inoue, J., 1996. TRAF5, a novel tumor necrosis factor receptor-associated factor family protein, mediates CD40 signaling. Proc. Natl. Acad. Sci. U.S.A. 93, 9437–9442.

Jenner, R.G., Alba, M.M., Boshoff, C., Kellam, P., 2001. Kaposi's sarcoma-associated herpesvirus latent and lytic gene expression as revealed by DNA arrays. J. Virol. 75, 891–902.

Joachims, M., Van Breugel, P.C., Lloyd, R.E., 1999. Cleavage of poly(A)-binding protein by enterovirus proteases concurrent with inhibition of translation in vitro. J. Virol. 73, 718–727.

Katano, H., Hoshino, Y., Morishita, Y., Nakamura, T., Satoh, H., Iwamoto, A., Herndier, B., Mori, S., 1999a. Establishing and characterizing a CD30-positive cell line harboring HHV-8 from a primary effusion lymphoma. J. Med. Virol. 58, 394–401.

Katano, H., Sata, T., Suda, T., Nakamura, T., Tachikawa, N., Nishizumi, H., Sakurada, S., Hayashi, Y., Koike, M., Iwamoto, A., Kurata, T., Mori, S., 1999b. Expression and antigenicity of human herpesvirus 8 encoded ORF59 protein in AIDS-associated Kaposi's sarcoma. J. Med. Virol. 59, 346–355.

Katano, H., Iwasaki, T., Baba, N., Terai, M., Mori, S., Iwamoto, A., Kurata, T., Sata, T., 2000a. Identification of antigenic proteins encoded by human herpesvirus 8 and seroprevalence in the general population and among patients with and without Kaposi's sarcoma. J. Virol. 74, 3478–3485.

Katano, H., Sato, Y., Kurata, T., Mori, S., Sata, T., 2000b. Expression and localization of human herpesvirus 8-encoded proteins in primary effusion lymphoma, Kaposi's sarcoma, and multicentric Castleman's disease. Virology 269, 335–344.

Katano, H., Suda, T., Morishita, Y., Yamamoto, K., Hoshino, Y., Nakamura, K., Tachikawa, N., Sata, T., Hamaguchi, H., Iwamoto, A., Mori, S., 2000c. Human herpesvirus 8-associated solid lymphomas that occur in AIDS patients take anaplastic large cell morphology. Mod. Pathol. 13, 77–85.

Katano, H., Ogawa-Goto, K., Hasegawa, H., Kurata, T., Sata, T., 2001. Human-herpesvirus-8-encoded K8 protein colocalizes with the promyelocytic leukemia protein (PML) bodies and recruits p53 to the PML bodies. Virology 286, 446–455.

Khanna, R., Kiledjian, M., 2004. Poly(A)-binding-protein-mediated regulation of hDcp2 decapping in vitro. EMBO J. 23, 1968–1976.

Kuyumcu-Martinez, N.M., Van Eden, M.E., Younan, P., Lloyd, R.E., 2004. Cleavage of poly(A)-binding protein by poliovirus 3C protease inhibits host cell translation: a novel mechanism for host translation shutoff. Mol. Cell. Biol. 24, 1779–1790.

Li, M., Lee, H., Guo, J., Neipel, F., Fleckenstein, B., Ozato, K., Jung, J.U., 1998. Kaposi's sarcoma-associated herpesvirus viral interferon regulatory factor. J. Virol. 72, 5433–5440.

Lubyova, B., Pitha, P.M., 2000. Characterization of a novel human herpesvirus 8-encoded protein, vIRF-3, that shows homology to viral and cellular interferon regulatory factors. J. Virol. 74, 8194–8201.

Lubyova, B., Kellum, M.J., Frisancho, A.J., Pitha, P.M., 2004. Kaposi's sarcoma-associated herpesvirus-encoded vIRF-3 stimulates the transcriptional activity of cellular IRF-3 and IRF-7. J. Biol. Chem. 279, 7643–7654.

Maul, G.G., 1998. Nuclear domain 10, the site of DNA virus transcription and replication. BioEssays 20, 660–667.

Minvielle-Sebastia, L., Preker, P.J., Wiederkehr, T., Strahm, Y., Keller, W., 1997. The major yeast poly(A)-binding protein is associated with cleavage factor IA and functions in premessenger RNA 3′-end formation. Proc. Natl. Acad. Sci. U.S.A. 94, 7897–7902.

Moore, P.S., Chang, Y., 2001. In: Knipe, D.M., Howley, P.M. (Eds.), Kaposi's Sarcoma-Associated Herpesvirus, 4th ed. Lippincott Williams and Wilkins, Philadelphia.

Pozharskaya, V.P., Weakland, L.L., Zimring, J.C., Krug, L.T., Unger, E.R., Neisch, A., Joshi, H., Inoue, N., Offermann, M.K., 2004. Short duration of elevated vIRF-1 expression during lytic replication of human herpesvirus 8 limits its ability to block antiviral responses induced by alpha interferon in BCBL-1 cells. J. Virol. 78, 6621–6635.

Rivas, C., Thlick, A.E., Parravicini, C., Moore, P.S., Chang, Y., 2001. Kaposi's sarcoma-associated herpesvirus LANA2 is a B-cell-specific latent viral protein that inhibits p53. J. Virol. 75, 429–438.

Sun, R., Lin, S.F., Staskus, K., Gradoville, L., Grogan, E., Haase, A., Miller, G., 1999. Kinetics of Kaposi's sarcoma-associated herpesvirus gene expression. J. Virol. 73, 2232–2242.

van der Kuyl, A.C., van den Burg, R., Zorgdrager, F., Dekker, J.T., Maas, J., van Noesel, C.J., Goudsmit, J., Cornelissen, M., 2002. Primary effect of chemotherapy on the transcription profile of AIDS-related Kaposi's sarcoma. BMC Cancer 2, 21.

Zimring, J.C., Goodbourn, S., Offermann, M.K., 1998. Human herpesvirus 8 encodes an interferon regulatory factor (IRF) homolog that represses IRF-1-mediated transcription. J. Virol. 72, 701–707.