主論文の要旨

Epstein-Barr Virus BKRF4 Gene Product Is Required for Efficient Progeny Production

（EB ウイルス BKRF4 遺伝子は効率のよい子孫ウイルス産生に必須である）

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【Background】

Epstein-Barr virus (EBV), a member of the herpesvirus family and double-stranded DNA virus, is ubiquitous, and infections are mostly asymptomatic or produce very mild symptoms. But in some cases, it can be associated with several malignancies, such as Burkitt lymphoma, nasopharyngeal carcinoma, and gastric carcinoma. It has two alternative stages of life cycle, latent and lytic, and is switched from the latent to the lytic form occasionally. While the latent genes of EBV and its association with lymphomagenesis are studied extensively, the roles of lytic cycle genes are poorly understood.

We here focused on one of such uncharacterized EBV lytic genes, *Bam*HI-K rightward reading frame 4 (BKRF4) and analyzed its function by preparing BKRF4 knockout viruses. In EBV research, bacterial artificial chromosome (BAC) system has been used to manipulate the virus genome, in combination with HEK293 cells, which is not a natural target cell type for EBV. Therefore, we have established a novel manipulation system, in which CRISPR/Cas9 system was used, so that we could analyze the behavior of the knockout virus in B cell background. By using knockout viruses prepared by both methods, we could successfully reveal that the BKRF4 gene product is involved in optimizing infectivity of progeny virus.

【Methods】

EBV BKRF4 gene was knocked out by BAC (B95-8 strain virus) and CRISPR/Cas9 (Akata strain virus) system, and analyzed the behavior in HEK293 and Akata cells, respectively. We carried out quantitative real-time RT-PCR (qRT-PCR), quantitative PCR (qPCR), immunoblotting (IB), fluorescence-activated cell sorting (FACS), immunoprecipitation (IP), and immunofluorescence (IF) analysis to figure out the functions of this gene product.

【Results】

Once EBV reactivated from latent to lytic, it expresses viral lytic genes in a sequential manner: immediate early, early, and late. In order to characterize the BKRF4, we first analyzed the kinetics of this gene using our affinity purified anti-serum against BKRF4 polypeptide. EBV positive B cells (B95-8) were lytically induced by TPA, A23187, sodium butyrate (TAB) treatment with or without PAA, the DNA polymerase inhibitor, to inhibit late viral gene expression. Using IB and qRT-PCR analysis, we found that the BKRF4 gene is expressed with late kinetics because its expression was suppressed by PAA (**Fig. 1A, B**). To study the role of BKRF4 in EBV lytic replication, we constructed the BKRF4-deficient and its revertant viruses (B95-8) in HEK293 cells using the BAC system (**Fig. 2A, B**) and investigated if it's required for the lytic replication of EBV (**Fig. 3**). Our results indicate that loss of BKRF4 did not affect EBV lytic gene expression (**Fig. 3A**) and viral DNA synthesis (**Fig. 3B**), but it markedly influenced the production of progeny and its infection efficiency (**Fig. 3C - G**).

Furthermore, we examined whether BKRF4 protein can associate with other viral proteins. We found that BKRF4 interacted with another EBV protein, BGLF2, through its C-terminal part (**Fig. 4**).

We also investigated whether an exogenous supply of BKRF4 *in trans* could complement the reduced production of infectious progeny in the knockout virus. Exogenous supply of wild-type BKRF4 fully complemented the reduced progeny levels in the knockout, whereas C-terminal deleted BKRF4 could only partially complement the progeny levels (**Fig. 5A**). The exogenous supply of the tagged vector was confirmed by immunoblot analysis (**Fig. 5B**). Thus, our result further confirms that BKRF4 is important for efficient production of infectious progeny virions and that the interaction between BKRF4 and BGLF2 through its conserved C-terminal motif plays an important role in efficient progeny production, at least in part (**Fig. 5**).

We next analyzed the subcellular localization of BKRF4 using different cell line (**Fig. 6 & 7**). Our investigation indicates that BKRF4 localized in the nucleus and perinuclear region in both infected (**Fig. 6**) and transfected (**Fig. 7**) condition, and the interaction partner BGLF2 was transported to the nucleus upon cotransfection with BKRF4 (**Fig. 7**).

Next, we prepared knockout virus of BKRF4 gene by CRISPR/Cas9 system to further confirm above results in B cells. Similar phenotype as in HEK293 (**Fig. 3**) was observed in the context of B cells; progeny production and infectivity was suppressed by disruption of the BKRF4 gene (**Fig. 8A - G**).

In addition, we revealed that BKRF4 is phosphorylated, and BGLF4, the only protein kinase of EBV may somehow be involved in the phosphorylation of BKRF4 (**Fig. 9A, B**).

Since BKRF4 has been suggested as a tegument protein, we also analyzed the presence of BKRF4 in the purified virion. BKRF4 appeared to be in the virion component, but it seemed associated with viral envelope or not attached very tightly to the nucleocapsid of the virion (**Fig. 10**).

【Discussion】

In this study, we have investigated the function of BKRF4, one of the lytic genes of EBV conserved among gammaherpesviruses but no homolog exist in alpha-or beta-herpesviruses. We found that the deficiency of BKRF4 had almost no effect on the viral gene expressions and DNA synthesis in HEK293 or Akata cells, but it significantly reduced the progeny virus production and its infectivity upon *de novo* infection. Our results coincide with those from a previous study on Kaposi's sarcoma herpesvirus (KSHV) ORF45 (Zhu, J Virol 2006), a homolog of EBV BKRF4. Most importantly, we could successfully analyze the functions of BKRF4 in B cells, the most natural host cell type for EBV. Editing the EBV lytic gene by CRISPR/Cas9 system sounds simple but cloning cells latently infected with the edited virus and triggering lytic induction from the latent cells was not easy.

We revealed that BKRF4 associated with another EBV protein, BGLF2 though its C-terminal residues. Notably, BKRF4 may play a crucial role in activating mitogen-activated protein kinase (MAPK) signaling through the interaction with BGLF2, since BGLF2 was recently found to activate AP-1 through activation of MAPK (Liu, J Virol 2015). In addition, EBV BGLF2 has an association with another myristoylated and palmitoylated tegument protein BBLF1 and plays crucial role in virion maturation (Chiu, J Virol 2012). Therefore, we are speculating that there is an interaction between BKRF4 and the membrane-anchored BGLF2/BBLF1 complex in the cytoplasm, which may contribute to efficient secondary envelopment.

We also found that BKRF4 protein can be phosphorylated and that the only EBV protein kinase, BGLF4, increased the phosphorylation either directly or indirectly. It has been reported that the homolog of BKRF4 in KSHV, ORF45, can also be phosphorylated (Zhu, J Virol 2003), and KSHV ORF36, the homolog of BGLF4, contributes to the phosphorylation (Avey, J Virol 2016). Although sequence homology between EBV BKRF4 and KSHV ORF45 is low, they likely share certain functions or regulatory mechanisms.

【Conclusion】

Our results demonstrated importance of the BKRF4 gene for the infectious progeny virions production, and shed light on the complicated process of EBV progeny maturation in the lytic cycle. Notably, this study is a successful example of disruption and characterization of an EBV lytic cycle gene in B cells with CRISPR/Cas9 technology. However, to clarify the underlying molecular mechanism, further investigation is warranted.