Assembly of the Epstein–Barr virus BBLF4, BSLF1 and BBLF2/3 proteins and their interactive properties

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Epstein–Barr virus (EBV) encodes putative helicase–primase proteins BBLF4, BSLF1 and BBLF2/3, which are essential for the lytic phase of viral DNA replication. The BSLF1, BBLF4 and BBLF2/3 proteins were expressed in B95-8 cells after induction of a virus productive cycle, possessing apparent molecular masses of 89 kDa, 90 kDa and 80 kDa, respectively. The anti-BSLF1 or anti-BBLF2/3 protein-specific antibody, which recognizes its target protein in both Western blotting and immunoprecipitation analyses, immunoprecipitated all of the BSLF1, BBLF4 and BBLF2/3 proteins from the extract of the cells with a virus productive cycle, indicating that these viral proteins are assembled together in vivo. To characterize their protein–protein interactions in detail, recombinant baculoviruses capable of expressing each of these viral gene products in insect cells were constructed. The assembly of the three virus replication proteins was reproduced in insect cells co-infected with the three recombinant baculoviruses, indicating that complex formation does not require other EBV replication proteins. Furthermore, experiments performed by using the extracts from insect cells co-infected with each pair of the recombinant viruses demonstrated that the BSLF1 protein could interact separately with both the BBLF4 and BBLF2/3 proteins and that the BBLF2/3 protein also interacted with the BBLF4 protein. These observations strongly suggest that within the BBLF4–BSLF1–BBLF2/3 complex each component interacts directly with the other two, resulting in helicase–primase enzyme activity.

Introduction

Epstein–Barr virus (EBV) is a human lymphotropic herpesvirus with a linear double-stranded DNA which is 172 kbp in length (Baer et al., 1984). Like other herpesviruses, EBV has both a latent state and a lytic replication cycle. In latently infected lymphoblastoid cells, the viral genome is maintained as a circular plasmid molecule and replicated by the replication machinery of the host cell (Yates & Guan, 1991; Aiyar et al., 1998). However, after induction of lytic virus replication, EBV DNA replication proteins are induced and the EBV genome is amplified 100- to 1000-fold via the lytic phase replication origin, oriLyt. The intermediate replication product is a large concatameric molecule in which the single genome units are arranged head to tail (Hammerschmidt & Sugden, 1988).

EBV encodes seven virus replication genes that are essential for oriLyt-dependent DNA replication (Fixman et al., 1992, 1995). The BLF1 protein is an oriLyt-binding protein and also acts as the lytic transactivator (Schepers et al., 1996). The BALF5 gene encodes the DNA Pol catalytic subunit (Tsurnumi et al., 1993a, b, 1994) and the BMRF1 gene encodes the DNA Pol accessory subunit (Kiehl & Dorsey, 1995; Tsurnumi, 1993; Tsurnumi et al., 1993a, b, 1994). A single-stranded DNA-binding protein is encoded by the BALF2 gene (Tsurnumi et al., 1996, 1998). The enzymatic activities of the remaining three proteins encoded by the BBLF4, BSLF1 and BBLF2/3 genes have not been demonstrated, but are predicted to act as helicase, primase and helicase–primase associated proteins, respectively (Fixman et al., 1995). The BBLF4 and BSLF1 gene products share 34% and 23% sequence identity with herpes simplex virus type 1 (HSV-1) UL5 and UL52 gene products and are composed of 810 and 875 amino acids (predicted Mr of 89 795 and 97 972, respectively. The BBLF2/3 gene product
has no significant similarity with the HSV-1 UL8 gene product in overall identity, but has a stretch of 55 amino acids which is similar to a region of the UL8 protein (Fixman et al., 1992). The BBLF2/3 gene product is translated from one spliced RNA derived from the two open reading frames (ORFs), BBLF2 and BBLF3, and is composed of 710 amino acids (predicted Mr of 78,176).

In the case of HSV-1, three essential virus replication proteins encoded by the UL5 (BBLF4 analogue), UL52 (BSLF1 analogue) and UL8 (BBLF2/3 analogue) genes are known to form a tripartite complex in both the HSV-1-infected cells and the insect cells triply infected with the recombinant baculoviruses capable of expressing these gene products (Crute et al., 1989; Dodson et al., 1989; McLean et al., 1994). The HSV-1 tripartite complex exhibits 5′ to 3′ DNA helicase, DNA primase and single-stranded DNA-dependent NTPase activities (Calder & Stow, 1990; Dodson & Lehman, 1991; Crute & Lehman, 1991; Crute et al., 1989; Dodson et al., 1989). Physical interaction between the UL5 and UL52 gene products has been demonstrated and the subassembly complex also exhibits all the enzymatic activities of the holoenzyme in vitro (Dodson & Lehman, 1991). The UL5 protein contains six conserved motifs that are found in all members of helicase superfamily I, which comprises DNA and RNA helicas from bacteria, viruses and eukaryotes (Zhu & Weller, 1992). The UL52 protein contains a DXD motif associated with catalytic activity in other primases (Dracheva et al., 1995; Klinedinst & Challberg, 1994). The UL8 protein has not been associated with any enzymatic activities but can stimulate both the helicase and primase activities of the subassembly complex. These results and mutational analyses suggest that the UL5 gene encodes the helicase subunit and that the UL52 gene encodes the primase subunit of the complex, although neither the UL5 nor the UL52 protein appears to possess any of these activities when expressed and purified alone (Dodson et al., 1989; Calder & Stow 1990; Dodson & Lehman, 1991).

Relatively few studies characterizing the EBV putative helicase–primase have been performed. More recently, Gao et al. (1998) expressed the three BBLF4, BSLF1 and BBLF2/3 proteins fused to a myc epitope in Vero cells by transfecting their expression vectors. When individually transfected, Myc–BBLF2/3 showed mixed nuclear and cytoplasmic staining, Myc–BSLF1 was perinuclear, and Myc–BBLF4 localized to the cytoplasm. Concurrent presence of the all three members resulted in nuclear localization of the BBLF4, BBLF2/3 and BSLF1 proteins. Thereby the authors supposed the presence of a BSLF1–BBLF4–BBLF2/3 complex in the transfected cells.

In this study we have demonstrated assembly of the BSLF1, BBLF4 and BBLF2/3 proteins in the EBV lytic phase-induced B cells, B95-8, by immunoprecipitation analyses with the BSLF1 or BBLF2/3 protein-specific antibody. Furthermore, we reproduced their complex formation in recombinant baculovirus expression systems. Experiments performed by double infections with each pair of the recombinant viruses revealed that subassemblies consisting of each component of the tripartite complex were observed, strongly suggesting that within the BBLF4–BSLF1–BBLF2/3 complex each component interacts directly with the other two.

**Methods**

- **Cells.** B95-8 cells, a mammalian lymphoblastoid cell line immortalized with human EBV, were grown at 37 °C in a 5% humidified CO₂ atmosphere in RPMI 1640 medium (Life Technologies) supplemented with 40 μg/ml kanamycin and 10% foetal calf serum (FCS). Spodoptera frugiperda (SF9) and Trichoplusia ni (High Five) insect cells were grown at 27 °C in SF-900 II SFM and Express Five SFM media (Life Technologies), respectively, supplemented with 20 μg/ml gentamicin and 10% FCS.

- **Construction of transfer vector plasmids.** A plasmid clone containing the EBV (Akata strain) BSLF1 ORF (Takada et al., 1991) was used to generate a transfer vector plasmid, pBSLF1/VI-L1392. The BantH–HindIII DNA fragment containing a full-length copy of the BSLF1 gene was subcloned into a plasmid pBlueScript (pBS) to generate plasmid pBSLF1/BS. Subsequently the BgII–XhoI DNA fragment containing a full copy of the BSLF1 gene was isolated from plasmid pBSLF1/BS and ligated between the BgII and XhoI sites of plasmid pVL1392 to generate pBSLF1/VI-L1392.

A cosmid clone containing the EBV (Akata strain) BBLF4 ORF (Takada et al., 1991) was used to generate a transfer vector plasmid, pBBLF4/VI-L1392. The EcoRI–HindIII DNA fragment containing a full-length copy of the BBLF4 gene was subcloned into cosmid pBl8 to generate cosmid pBBLF4/IB8. The PsI–HindIII DNA fragment containing a downstream part of the BBLF4 gene was isolated from the cosmid pBBLF4/IB8 and inserted into the PsI and HindIII sites of the pBS to generate plasmid pBBLF4/LS. Next, the PsI–PsI fragment containing an upstream part of the BBLF4 gene was isolated from the cosmid pBBLF4/IB8 and ligated at the PsI site of the plasmid pBBLF4/LS to generate plasmid pBBLF4/LS, consequently containing a full copy of the EBV BBLF4 gene. The pBBLF4/LS was digested with NcoI, blunt-ended, and subsequently digested with XhoI. The NcoI (blunt-ended)–XhoI DNA fragment containing a full copy of the BBLF4 gene was isolated and ligated between the XhoI and SalI sites of pVL1392 to generate pBBLF4/VI-L1392.

A plasmid, pEF76A, containing a full-length spliced BBLF2/3 cDNA (Fixman et al., 1995) was used to generate a recombinant donor plasmid, pBBLF2/3/FBD. The DNA fragment encoding the intact BBLF2/3 gene was amplified by PCR with a pair of primers, BBLF2/3-Bam and BBLF2/3-Eco. The primers sequences were as follows: BBLF2/3-Bam, 5′-aaagatCCCTCAGATCTCGATAAAGAAGATGGGAGAAACGGC3′; BBLF2/3-Eco, 5′-aaagatctCTCTCTAATAAATTTCTTCTAGATATAAATGAGG3′. The DNA fragments amplified with the primer pair were digested with BamH1 and EcoRI and ligated between the BamH1 and EcoRI sites of a Bac-to-Bac donor plasmid, pFastBac DUAL (Life Technologies), to generate pBBLF2/3/FBD.

- **Construction of the recombinant baculovirus expressing the BBLF4, BSLF1 or BBLF2/3 gene product.** The transfer vector plasmid, pBBLF4/VI-L1392 or pBSLF1/VI-L1392, was co-transfected with the Snol-digested linear baculovirus (AcRP23-lacZ) DNA into Sf9 cells as described previously (Kitts et al., 1990). Viruses propagated from the transfected cells were used to infect Sf9 cells and selected by staining with 50 μg/ml neutral red and 250 μg/ml X-Gal at 3 days post-infection. The plaques formed by the recombinant baculoviruses are distinguishable from those of the original baculoviruses by the lack of blue colour in the
infected cells. The recombinant baculoviruses were plaque-purified three times and designated as AcBBLF4 and AcBBLF3, respectively.

The recombinant donor plasmid pBBLF2/3/FBD was transformed into DH10Bac competent cells (Life Technologies). In the cells, the mini-Tn7 element in the donor plasmid can transpose to the mini-attTn7 target site on the bacmid, which is a recombinant infectious baculovirus DNA containing a mini-F replicon, a kanamycin resistance marker, and the attTn7 target site (Luckow et al., 1993). The resulting transposed bacmid containing a full copy of the BBLF2/3 gene was transfected into Sf9 cells and a recombinant baculovirus, AcBBLF2/3, was obtained from the transfected cells. Details of the technical methods were according to the instruction manual of the Bac-to-Bac Baculovirus expression system (Life Technologies).

**Antibodies.** Two kinds of anti-BSLF1 protein-specific rabbit antibodies were raised against two synthetic proteins, a T7 phage f10 protein fused with each of two truncated regions of the BSLF1 protein separately. One is a carboxy-terminal domain (504 amino acids) and the other is a central domain derived from a Pst–PstI fragment in the BSLF1 ORF (547 amino acids). These fusion proteins were prepared by using an *E. coli* expression system and SDS–PAGE purification as described previously (Tsurumi et al., 1993a). These rabbit antibodies were designated as anti-BSLF1c and anti-BSLF1p protein-specific antibodies, respectively. Immunoglobulin G (IgG) from the immunized rabbit serum was purified as described previously (Tsurumi et al., 1993a).

Anti-BBLF4 protein-specific rabbit antibody was raised against a BBLF4 carboxy-terminal oligopeptide (20 amino acids) coupled to keyhole limpet haemacyanin. Preparation for the anti-BBLF4 protein-specific IgG was performed as described previously (Tsurumi et al., 1993a).

Two kinds of anti-BBLF2/3 protein-specific rabbit antibodies were prepared. One was raised against a BBLF2/3 carboxy-terminal oligopeptide (20 amino acids) coupled to keyhole limpet haemacyanin and another was raised against a whole BBLF2/3 gene product which was purified from the AcBBLF2/3 recombinant baculovirus-infected High Five insect cells. These rabbit antibodies were designated as anti-BBLF2/3c and anti-BBLF2/3w protein-specific antibodies, respectively. Preparations for these antibodies were performed according to the same procedure as described previously (Tsurumi et al., 1993a).

**Preparation of the lysate from B95-8 cells with a virus productive cycle.** B95-8 cells were seeded at approximately 3 × 10^5 cells/ml in 30 ml culture medium with 200 ng/ml phorbol 12-myristate 13-acetate (TPA), 5 mM sodium n-butyrate and 1 mM calcium ionophore A23187 for induction of the EBV lytic replication phase. At 72 h post-induction, the cells were harvested and washed with cold PBS. The cell pellet was resuspended in 5 ml hypotonic buffer (50 mM Tris–HCl pH 7.6, 1 mM EDTA, 1 mM PMSF, 1 mM DTT and 10 µg/ml each of pepstatin A and leupeptin), stored on ice for 30 min, and then centrifuged at 1500 g for 10 min at 4°C. The clarified lysate was confirmed to contain the BSLF1, BBLF4 and BBLF2/3 proteins as judged by Western blotting analyses using each of the protein-specific antibodies, frozen in liquid nitrogen and then stored at −80 °C until use.

**Preparation of the cell extracts from High Five cells infected with the recombinant baculoviruses.** High Five cells were seeded at approximately 1 × 10^6 cells per well in 6-well plates and infected or co-infected with the indicated recombinant baculovirus(es) at an m.o.i. of 5 p.f.u. per cell for each virus. At 72 h post-infection the cells were harvested and washed three times with cold PBS. The cells were resuspended in 1 ml lysis buffer (50 mM Tris–HCl pH 8.0, 300 mM NaCl, 1% NP-40, 1 mM PMSF, 1 mM DTT and 10 µg/ml each of pepstatin A and leupeptin) and incubated on ice for 20 min. These lysates were centrifuged at 18,000 g for 10 min at 4 °C and the supernatants were frozen in liquid nitrogen and then stored at −80 °C until use in immunoprecipitation analyses.

**Immunoprecipitation analyses.** These were performed according to standard procedures (Sambrook et al., 1989). Briefly, the cell extracts described above were equally divided into microfuge tubes and the aliquots were mixed with 3 µg of the anti-BSLF1c or the anti-BBLF2/3w protein-specific antibody (IgG) or the control rabbit IgG (DAKO), and gently rocked for 1 h at 4 °C. After the addition of 20 µl protein A–agarose beads (Pharmacia Biotech), the mixtures were further incubated for 1 h at 4 °C, rocking gently. The tertiary protein A–antibody–antigen complexes were collected by centrifugation at 18,000 g for 1 min at 4 °C, resuspended in 1 ml NET gel buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.1% NP-40 and 1 mM EDTA), and then incubated for 15 min at 4 °C, rocking gently. This washing step was repeated three times. Each of the final precipitated beads was suspended in 60 µl SDS-sample buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.01% bromphenol blue) and heated at 100 °C for 6 min. After centrifugation at 18,000 g for 5 min, the protein A–agarose beads were removed and the supernatants were subjected to SDS–PAGE using a 10% polyacrylamide gel and Western blotting analyses, performed as described previously (Fuijita et al., 1998). Briefly, the protein blots transferred onto a nitrocellulose membrane were incubated with the indicated first antibodies for 30 min at 37 °C, subsequently probed with peroxidase-labelled goat anti-rabbit IgG for 30 min at 37 °C, and then visualized by using an ECL (Amersham Pharmacia).

**Results**

**Detection of assembly of the EBV BBLF4, BSLF1 and BBLF2/3 proteins in B95-8 cells**

To investigate whether the EBV BBLF4, BSLF1 and BBLF2/3 proteins form a complex in B95-8 cells with a virus productive cycle, immunoprecipitations combined with Western blotting analyses were carried out. We prepared five antibodies specific for each of the three viral proteins for Western blotting analyses. Among them, anti-BSLF1c and anti-BBLF2/3w protein-specific antibodies could precipitate their target proteins effectively and had no ability to precipitate the other viral proteins, indicating that these antibodies are effective for immunoprecipitation analyses. As a negative control, a normal rabbit IgG was used.

The EBV lytic phase of DNA replication was induced by treatment with a combination of chemical agents such as TPA, sodium n-butyrate and calcium ionophore A23187. Before induction, the BSLF1 protein was not detected, but small amounts of the BBLF4 and the BBLF2/3 proteins were expressed in the B95-8 cells as judged by Western blotting analyses (data not shown). It was known that about 5% of the B95-8 cells in the culture media were in the lytic phase. After the induction, the amounts of the BSLF1, BBLF4 and BBLF2/3 proteins in the cells gradually increased and reached a peak at 72 h post-induction. The molecular masses of the BSLF1,
Fig. 1. Detection of the EBV BBLF4–BSLF1–BBLF2/3 complex in B95-8 cells with a virus productive cycle by co-immunoprecipitation with anti-BSLF1c specific antibody. The lytic phase of EBV DNA replication was induced in B95-8 cells by treatment with TPA, sodium n-butyrate and calcium ionophore. The cells were harvested at 72 h post-induction and the clarified lysate was prepared as described in Methods. The cell extracts were subjected to immunoprecipitation (I.P.) analyses by using the anti-BSLF1c specific IgG (Anti-BSLF1) or the control rabbit IgG. The cell extracts were mixed with 3 µg of the anti-BSLF1c specific antibody (IgG) or the control rabbit IgG, and gently rocked for 1 h at 4 °C. After addition of 20 µl protein A–agarose beads, the mixtures were further incubated for 1 h at 4 °C, centrifuged at 18000 g for 1 min at 4 °C, and washed with 1 ml NET gel buffer (50 mM Tris–HCl pH 8–0, 150 mM NaCl, 0–1% NP-40 and 1 mM EDTA). This washing step was repeated three times. Immunoprecipitated proteins were resolved by SDS–PAGE (10% polyacrylamide) and analysed by Western blotting with anti-BSLF1p (a), anti-BBLF4 (b) or anti-BBLF2/3w (c) specific antibody. Lane 3 of each panel shows the input sample before immunoprecipitation. The positions of the molecular mass standards (in kDa) are indicated on the left side of each panel. Immunoprecipitated proteins are indicated by arrowheads.

BBLF4 and BBLF2/3 proteins expressed in the B95-8 cells were 89 kDa, 90 kDa and 80 kDa, respectively, as determined by mobilities in SDS–PAGE. The clarified lysates were shown to contain these virus replication proteins by Western blotting analyses (data not shown) and were subjected to immunoprecipitation analysis with the anti-BSLF1c or anti-BBLF2/3w specific antibody (IgG). The immunoprecipitated proteins were subjected to SDS–PAGE, transferred onto a nitrocellulose membrane, and then the protein blots were incubated with the indicated first antibodies.

The results from the immunoprecipitation analysis using the anti-BSLF1c specific IgG are shown in Fig. 1. The control rabbit IgG could not precipitate any of these viral proteins from the cell extract. In contrast, the anti-BSLF1c specific IgG not only precipitated the corresponded 89 kDa BSLF1 protein (Fig. 1a), but also co-precipitated the 90 kDa BBLF4 and 80 kDa BBLF2/3 proteins (Fig. 1b, c) from the clarified cell extract. As shown in Fig. 4, the anti-BSLF1c specific IgG cannot precipitate the BBLF4 and BBLF2/3 proteins directly, although the IgG can precipitate the corresponding BSLF1 protein. Fig. 2 shows immunoprecipitation analysis using the anti-BBLF2/3w specific IgG. The IgG cannot precipitate the BBLF4 protein (see Fig. 6) and the BSLF1 protein (data not shown) directly. However, the BBLF2/3w specific IgG co-immunoprecipitated the BBLF4 and BSLF1 proteins (Fig. 2a, b) in addition to the BBLF2/3 protein (Fig. 2c) from the cell extract. These results indicate that the EBV BBLF4, BSLF1 and BBLF2/3 proteins are assembled together in the B95-8 cells after induction of the lytic phase of the EBV DNA replication. It would seem that these virus replication proteins are assembled through specific protein–protein interactions and/or through interactions with viral DNA or with other virus replication proteins.

**Overexpression of the EBV BBLF4, BSLF1 and BBLF2/3 proteins**

In order to characterize the protein–protein interactions in detail, we constructed recombinant baculoviruses which express the products of each of these three genes in the infected insect cells.

Monolayers of High Five cells were mock-infected or infected at an m.o.i. of 5 p.f.u. per cell with each recombinant baculovirus, AcBBLF4, AcBSLF1 or AcBBLF2/3. The whole cell lysates were resolved by SDS–PAGE (10% polyacrylamide gel). In the AcBBLF4-infected cells, a 90 kDa polypeptide was
Assembly of EBV helicase–primase

Fig. 2. Detection of the EBV BBLF4–BSLF1–BBLF2/3 complex in B95-8 cells with a virus productive cycle by co-immunoprecipitation with anti-BBLF2/3w specific antibody. The lytic phase of EBV DNA replication was induced in B95-8 cells by treatment with TPA, sodium n-butyrate and calcium ionophore. The cells were harvested at 72 h post-induction and the clarified lysate was prepared as described in Methods. The cell extracts were subjected to immunoprecipitation (I. P.) analyses by using the anti-BBLF2/3w specific IgG (Anti-BBLF2/3) or the control rabbit IgG. The cell extracts were mixed with 3 μg of the anti-BBLF2/3w specific antibody (IgG) or the control rabbit IgG, and gently rocked for 1 h at 4 °C. After addition of 20 μl protein A–agarose beads, the mixtures were further incubated for 1 h at 4 °C, centrifuged at 18,000 g for 1 min at 4 °C, and washed with 1 ml NET gel buffer (50 mM Tris–HCl pH 8–0, 150 mM NaCl, 0–1% NP-40 and 1 mM EDTA). This washing step was repeated three times. Immunoprecipitated proteins were resolved by SDS–PAGE (10% polyacrylamide) and analysed by Western blotting with anti-BSLF1p (a), anti-BBLF4 (b) or anti-BBLF2/3w (c) specific antibody. Lane 3 of each panel shows the input sample before immunoprecipitation. Immunoprecipitated proteins are indicated by arrowheads.

Fig. 3. Overexpression of the BBLF4 (a), BSLF1 (b) or BBLF2/3 (c) gene products in High Five insect cells. High Five cells were mock-infected (M) or infected with each of the recombinant baculoviruses, AcBBLF4 (4), AcBSLF1 (1) and AcBBLF2/3 (2/3), and harvested at 72 h post-infection. Proteins recovered from the whole cell extracts were resolved by SDS–PAGE (10% polyacrylamide) and stained with Coomassie blue (left panel) or analysed by Western blotting with anti-BBLF4 (a), anti-BSLF1p (b) or anti-BBLF2/3c (c) specific antibody (right panels). The positions of the molecular mass standards (in kDa) are indicated on the left side of the left panels. The position of each of the gene products is indicated by an arrowhead on the right side of each panel.

overproduced and the expressed protein was identified as the EBV BBLF4 gene product by its reaction with the anti-BBLF4 protein-specific antibody in Western blotting analysis (Fig. 3a). A more weakly signalling 97 kDa polypeptide, in addition to the 90 kDa protein, was also detected by the anti-BBLF4 protein-specific antibody. In the AcBSLF1-infected cells, a 89 kDa polypeptide was overproduced and identified as the EBV BSLF1 gene product in Western blotting analysis by using the anti-BSLF1p specific antibody (Fig. 3b). The mobilities of the synthesized BSLF1 and BBLF4 proteins in the SDS–PAGE were similar to those of these viral proteins expressed in the B95-8 cells (data not shown). In the AcBBLF2/3-infected cells, a 76 kDa polypeptide was overproduced and identified as the EBV BBLF2/3 gene product in Western blotting analysis by using the anti-BBLF2/3c specific antibody (Fig. 3c). Although the overexpressed recombinant protein accounted for up to 10% of the total protein in each of the recombinant baculoviruses-infected cells, less than 5% of the expressed proteins were soluble.

Reproduction of the assembly of the EBV BBLF4, BSLF1 and BBLF2/3 proteins in High Five insect cells

To confirm whether the assembly of the BBLF4, BSLF1 and BBLF2/3 proteins is reproduced in insect cells and to deny the possibility that other EBV replication proteins are involved in
the complex formation, High Five insect cells were singly or triply infected with the AcBBLF4, AcBSLF1 and AcBBLF2/3 recombinant baculoviruses, and immunoprecipitation analyses were performed. The cells were harvested at 72 h post-infection and the clarified cell lysates were prepared. As shown in Fig. 4, the anti-BSLF1c specific IgG can immunoprecipitate the corresponded BSLF1 protein, but does not react with the other BBLF4 and BBLF2/3 proteins. When the insect cells were triply infected with the three recombinant baculoviruses, the anti-BSLF1c specific IgG precipitated all of the BSLF1, BBLF4 and BBLF2/3 proteins. The control rabbit IgG could not precipitate these proteins. These results indicate that co-precipitation of the BBLF4 and BBLF2/3 proteins is due to the presence of the BSLF1 protein and that the assembly is performed only among these three replication proteins.

**Subassemblies of each component of the BSLF1–BBLF4–BBLF2/3 complex**

In order to know how these three proteins interact to form a tripartite complex, their sectional interactions were examined. The successful overexpression of the tripartite complex in triply infected cells suggests that subassemblies of the proteins might be generated from doubly infected cells. Extracts from the insect cells doubly infected with AcBSLF1 and either AcBBLF4 or AcBBLF2/3 were prepared and subjected similarly to the immunoprecipitation analyses by using the anti-BSLF1c specific IgG (Fig. 5). The antibody could co-precipitate the BBLF4 and BBLF2/3 proteins separately. Thus, the BBLF4 and BBLF2/3 proteins could each interact separately with the BSLF1 protein. Furthermore, as shown in Fig. 6, when the cells were infected with AcBBLF4 and AcBBLF2/3, the anti-BBLF2/3w specific IgG precipitated the BBLF4 protein as well as the BBLF2/3 protein, although the antibody cannot react directly with the BBLF4 protein (Fig. 6b). Thus, we could demonstrate BSLF1–BBLF4, BSLF1–BBLF2/3 and BBLF4–BBLF2/3 subassemblies in the doubly infected insect cells. These results strongly suggest that within the BBLF4–

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**Fig. 4.** A tripartite complex formation of the EBV BBLF4, BSLF1 and BBLF2/3 proteins in High Five insect cells. High Five cells were infected triply with the recombinant baculoviruses, AcBBLF4, AcBSLF1 and AcBBLF2/3 (T), or singly with AcBBLF4 (4) or AcBBLF2/3 (2/3) and harvested at 72 h post-infection. The cell extracts were prepared as described in Methods and were subjected to immunoprecipitation (I. P.) analyses by using either the anti-BSLF1c specific IgG (Anti-BSLF1) or the control rabbit IgG. Immunoprecipitated proteins were resolved by SDS–PAGE (10% polyacrylamide) and analysed by Western blotting with anti-BSLF1p (a), anti-BBLF4 (b) or anti-BBLF2/3c (c) specific antibody. Immunoprecipitated proteins are indicated by arrowheads.

**Fig. 5.** Complex formations between the BSLF1 and BBLF4 proteins (a) and between the BSLF1 and BBLF2/3 proteins (b) in High Five insect cells. High Five cells were doubly infected with AcBSLF1 and either AcBBLF4 (1&4) or AcBBLF2/3 (1&2/3) and harvested at 72 h post-infection. Each of the cell extracts was subjected to immunoprecipitation (I. P.) analyses by using either the anti-BSLF1c specific IgG (anti-BSLF1) or the control rabbit IgG as described in Methods. Immunoprecipitated proteins were resolved by SDS–PAGE (10% polyacrylamide) and analysed by Western blotting with anti-BBLF4 (a) or anti-BBLF2/3c (b) specific antibody. Immunoprecipitated proteins are indicated by arrowheads.
BSLF1–BBLF2/3 complex each component interacts directly with the other two.

**Discussion**

Lytic EBV replication can be induced by treatment of the latently infected cells with various chemical agents (zur Hausen et al., 1978; Faggioni et al., 1986). Induction levels of the BSLF1, BBLF4 and BBLF2/3 proteins in lytic phase-induced B95-8 cells were low compared with those of the Balf2 protein or the BMRF1 protein. Especially the expression level of the BSLF1 protein was very low and sometimes undetectable in Western blotting analysis. So, we investigated the optimal condition for induction of lytic phase of B95-8 cells by using a combination of three chemical inducers: protein kinase C stimulator TPA, cell cycle blocker sodium n-butyrate, and calcium ionophore A23187. It turned out that a concentration of 200 ng/ml for TPA, 5 mM for sodium n-butyrate and 1 µM for calcium ionophore in the culture medium was the most effective for induction of the BSLF1 protein in B95-8 cells.

Gao et al. (1998) have supposed the presence of the BSLF1–BBLF4–BBLF2/3 complex through immunofluorescence assays with their nuclear re-translocations. When the expression vectors capable of expressing the BBLF4, BSLF1 and BBLF2/3 proteins fused to the myc epitope were individually transfected, Myc–BSLF1 protein showed perinuclear staining and Myc–BBLF2/3 protein showed mixed nuclear and cytoplasmic staining. Myc–BBLF4 protein localized to the cytoplasm. However, when the BBLF2/3 vector was co-transfected with the BSLF1 vector, the localization pattern changed to that of Myc–BSLF1 and was strictly cytoplasmic. Triple transfection of BBLF4, BBLF2/3 and BSLF1 vectors resulted in nuclear localization of these proteins. Thereby the authors expected the presence of a BSLF1–BBLF4–BBLF2/3 complex. In this study, the anti-BSLF1 protein or the anti-BBLF2/3 protein-specific antibody co-precipitated all of the BSLF1, BBLF4 and BBLF2/3 proteins from the extract of B95-8 cells with an EBV productive cycle. Thus, we directly demonstrated and confirmed the assembly of BBLF4, BSLF1 and BBLF2/3 proteins in the lytic phase of B95-8 cells.

We designed baculovirus expression systems for the BBLF4, BSLF1 and BBLF2/3 proteins to characterize the direct protein–protein interactions among these proteins in the infected insect cells. We reproduced their complex formation in the recombinant baculovirus expression systems. Experiments performed by the double infection with each pair of the recombinant viruses revealed that within the BBLF4– BSLF1–BBLF2/3 complex each component interacts directly with the other two. The clarified lyses for immunoprecipitation analyses were prepared from the insect cells triple or doubly infected with recombinant baculoviruses in the presence of 1% NP-40 and 300 mM NaCl. Since these assemblies were resistant to the detergent and high salt condition, the tripartite complex and the subassemblies observed appear to be stable.

The BBLF2/3 gene product expressed in B95-8 cells was an 80 kDa protein. On the other hand, the BBLF2/3 gene product in High Five insect cells had a molecular mass of 76 kDa. These polypeptides were recognized solely by the anti-BBLF2/3n antibody, which is raised against an N-terminal oligopeptide (20 amino acids) of the BBLF2 protein, and by the anti-BBLF2/3c antibody, which is raised against a C-terminal oligopeptide (20 amino acids) of the BBLF3 protein (data not shown). Thus the 80 kDa polypeptide expressed in B95-8 cells seems to be translated from one spliced RNA derived from the two ORFs, BBLF2 and BBLF3, as has been stated in a review (Farrell, 1989). At present we cannot explain the difference in the molecular masses of the expressed proteins in B95-8 cells and High Five cells. The expression plasmid to be used for the construction of AcBBLF2/3 is demonstrated to be able to support oriLyt replication (Fixman et al., 1995). The plasmid contains the synthetic BBLF2/3 cDNA, which is derived from M-ABA strain BBLF2 and BBLF3 ORFs and the Akata strain 191 bp PCR product. It remains to be determined whether the difference in the molecular masses is due to the difference in nucleotide sequences between the strains or due to the difference in post-translational modification.

The BBLF4 gene product shares 34% amino acid sequence identity with the HSV-1 UL5 gene product. Further, the BBLF4 gene product possesses the six conserved motifs found in all members of helicase superfamily I, as well as the HSV-1 UL5 gene product (Graves-Woodward & Weller, 1996; Spector et al., 1998; N. Yokoyama & T. Tsurumi, unpublished result). Substitutions of the conserved residues in each of the six
helicase motifs of the HSV-1 UL5 protein abolished the ability of UL5 to support viral DNA replication in vivo (Zhu & Weller, 1992) and the helicase activity of the purified UL5–UL52 subcomplex in vitro, but not the primase activity (Graves-Woodward et al., 1997). The BSLF1 gene product shares 23% amino acid sequence identity with the HSV-1 UL52 gene product and contains five regions conserved among other identified herpesvirus UL52 homologues, including a DXD motif which resembles the putative metal-binding site found in other primases (Dracheva et al., 1995; Klinedinst & Challberg, 1994). Changing either of the two aspartate residues in the primase DXD motif of the HSV-1 UL52 gene product abolished the primase activity of the purified UL5–UL52–UL8 complex, but not the ATPase and helicase activities (Dracheva et al., 1995; Klinedinst & Challberg, 1994). The BBLF2/3 gene product has no significant similarity with the HSV-1 UL8 gene product in overall identity but has a stretch of 55 amino acids which is similar to the UL8 gene product. Unlike the case of the HSV-1 UL8 protein, the BBLF2/3 protein has a potential ATP-binding motif, the function of which is unclear (Fixman et al., 1992). Although the UL8 gene product is not absolutely required for the helicase and primase activities in vivo, it interacts with the UL5–UL52 subcomplex and is essential for viral DNA replication in vivo. These sequence homologies between the HSV-1 and EBV gene products encourage further analyses to identify enzymatic activities of the EBV tripartite complex.

We constructed another recombinant baculovirus (AcBBLF2/3-Ht) expressing a BBLF2/3 protein fused with a polyhistidine tag at its N-terminal site in order to partially purify the BSLF1–BBLF4–BBLF2/3–Ht complex. HighFive cells were triply infected with AcBSLF1, AcBBLF4 and AcBBLF2/3–Ht and the clarified cell lysate was subjected to metal affinity chromatography. The tagged BBLF2/3, the BBLF4 and BSLF1 proteins were co-purified with approximately 1:1:1 stoichiometry through the column (data not shown). Therefore, the BBLF4–BSLF1–BBLF2/3 complex appears to be purified via the tagged BBLF2/3 protein. These observations further confirmed that the EBV BBLF4, BSLF1 and BBLF2/3 proteins form a tripartite complex. Although the BBLF4–BSLF1–BBLF2/3–Ht tripartite complex purified partially by the metal affinity chromatography exhibited DNA-dependent ATPase and primase activities, the control sample purified from the parent baculovirus (AcMNPV)-infected insect cells also showed these activities to some extent (data not shown). In order to determine whether the helicase and primase activities are really associated with the BBLF4–BSLF1–BBLF2/3 tripartite complex, further purification is needed and is now in progress.

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Assembly of EBV helicase–primase


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