JOURNAL OF VIROLOGY, Nov. 1996, p. 8047–8054 Vol. 70, No. 11
0022-538X/96/$04.00+0


# Two 21-Kilodalton Components of the Epstein-Barr Virus Capsid Antigen Complex and Their Relationship to ZEBRA-Associated Protein p21 (ZAP21)

TRICIA R. SERIO,[1] ANTONIO ANGELONI,[2]† JOHN L. KOLMAN,[1]‡ LYNDLE GRADOVILLE,[2] REN SUN,[1,3] DAVID A. KATZ,[1]§ WOUT VAN GRUNSVEN,[4] JAAP MIDDELDORP,[4] AND GEORGE MILLER[1,2,5]*

*Departments of Molecular Biophysics and Biochemistry,[1] Pediatrics,[2] Genetics,[3] and Epidemiology and Public Health,[5] Yale University, New Haven, Connecticut 06520, and Organon Teknika, Boxtel, The Netherlands[4]*

Received 11 June 1996/Accepted 6 August 1996

**The viral capsid antigen complex of Epstein-Barr virus (EBV), an important serodiagnostic marker of infection with the virus, consists of at least four components, with molecular masses of 150, 110, 40, and 21 kDa. Here we show that the 21-kDa component of the viral capsid antigen consists of products of two EBV genes, BFRF3 and BLRF2. Both products were expressed from late transcripts, were recognized by human antisera, and were present in virions. The BFRF3 product, but not that of BLRF2, fulfilled the definition of ZEBRA-associated protein p21 (ZAP21). In cells in which EBV was lytically replicating, BFRF3 protein was coimmunoprecipitated together with ZEBRA by a rabbit antiserum directed against amino acids 197 to 245 of BZLF1. In EBV-negative cells cotransfected with BZLF1 and BFRF3 expression vectors, BFRF3 was also coimmunoprecipitated with this antiserum. Although this antiserum could not detect BFRF3 on an immunoblot, it was able to immunoprecipitate BFRF3 in the absence of ZEBRA expression. The rabbit antiserum to amino acids 197 to 245 of BZLF1 was found to detect the same epitope at the carboxy end of BFRF3 as was recognized by rabbit antiserum to BFRF3 itself. Thus, coimmunoprecipitation of BFRF3 p21 with ZEBRA appeared to be due to cross-reactivity of the immunoprecipitating antiserum rather than to direct association of ZEBRA and BFRF3 p21.**

Nonenvelope virion components of Epstein-Barr virus (EBV) have not been extensively characterized, largely as the result of the lack of a fully lytic permissive system in vitro. These proteins are of obvious importance for a detailed understanding of virion assembly. Virion components form the basis of the classical immunofluorescence tests for viral capsid antigen (VCA). Assays for VCA were seminal in early experiments demonstrating that only a subpopulation of EBV-infected cells were activated into the lytic cycle (6). Moreover, assays for antibodies to VCA in human serum specimens permitted seroepidemiologic surveys which were instrumental in providing evidence for an etiologic association between EBV and Burkitt's lymphoma, nasopharyngeal carcinoma, and infectious mononucleosis.

VCA is a complex of incompletely defined EBV late-gene products which are associated with virion particles. Four genes encoding components of this complex have been identified as virion associated, although not all of them are capsid components. BcLF1 encodes the major capsid antigen of EBV, a nonglycosylated 150- to 160-kDa protein (4, 8, 26). BALF4 encodes a 110- to 125-kDa glycoprotein that is homologous to glycoprotein B of herpes simplex virus type 1 and glycoprotein II of varicella-zoster virus. Protein homologs of BALF4 are associated with the tegument (5, 10, 21). BALF4 is thought to be a major immunogen of VCA (13). BdRF1 encodes a 40-kDa protein which was recently characterized as a diagnostically relevant marker antigen (23, 25). BFRF3 encodes an 18- to 21-kDa protein which is another immunodominant marker for EBV infection (23–25).

Our interest in EBV p21 late proteins derived from experiments of Katz et al., who described a 21-kDa protein (ZEBRA-associated protein p21 [ZAP21]) which was coimmunoprecipitated with ZEBRA by certain rabbit antisera raised to portions of the BZLF1 gene product (9). The pattern of expression and immunoreactivity of ZAP21 indicated that it was a late viral protein. Moreover, ZAP21 was located in EBV virions, from which it could be immunoprecipitated by anti-ZEBRA serum. One candidate for ZAP21 was the gene BLRF2, which was identified by a computer survey of EBV late open reading frames (ORFs) predicted to encode proteins of the correct size (2, 3). The BLRF2 protein had not been previously characterized. Another candidate was the recently described BFRF3 component of VCA (25), which had been previously misclassified as an early protein (7). In this study, we demonstrate that BLRF2 is a component of the late p21 complex and we investigate the relationship of BFRF3 and BLRF2 to ZAP21.

## MATERIALS AND METHODS

**Cells.** Lymphocyte cell lines were grown in RPMI 1640 medium supplemented with 8% fetal bovine serum. BJAB is an EBV-negative human B-cell line derived from a B-cell lymphoma (14). Raji is a human B-cell line derived from a Burkitt's lymphoma harboring a defective EBV which is unable to replicate viral DNA or express late viral genes (17). P3J-HR-1 clone 16 (HH514-16) is a clonal human B-cell line derived from an EBV-positive Burkitt's lymphoma (18). B95-8 is a marmoset B-cell line transformed with EBV (16). LTK$^-$, a mouse fibroblast cell line (11), was maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum.

**Chemical induction.** Lymphocyte cell lines were split 6 days prior to harvest-

* Corresponding author. Mailing address: Departments of Molecular Biophysics and Biochemistry, Pediatrics, and Epidemiology and Public Health, Yale University, 333 Cedar St., New Haven, CT 06520.

† Present address: Department of Experimental Medicine, Policlinico Umberto I, Università Degli Studi di Roma "La Sapienza", Rome, Italy.

‡ Present address: The Salk Institute, La Jolla, Calif.

§ Present address: Abbott Laboratories, Abbott Park, Ill.

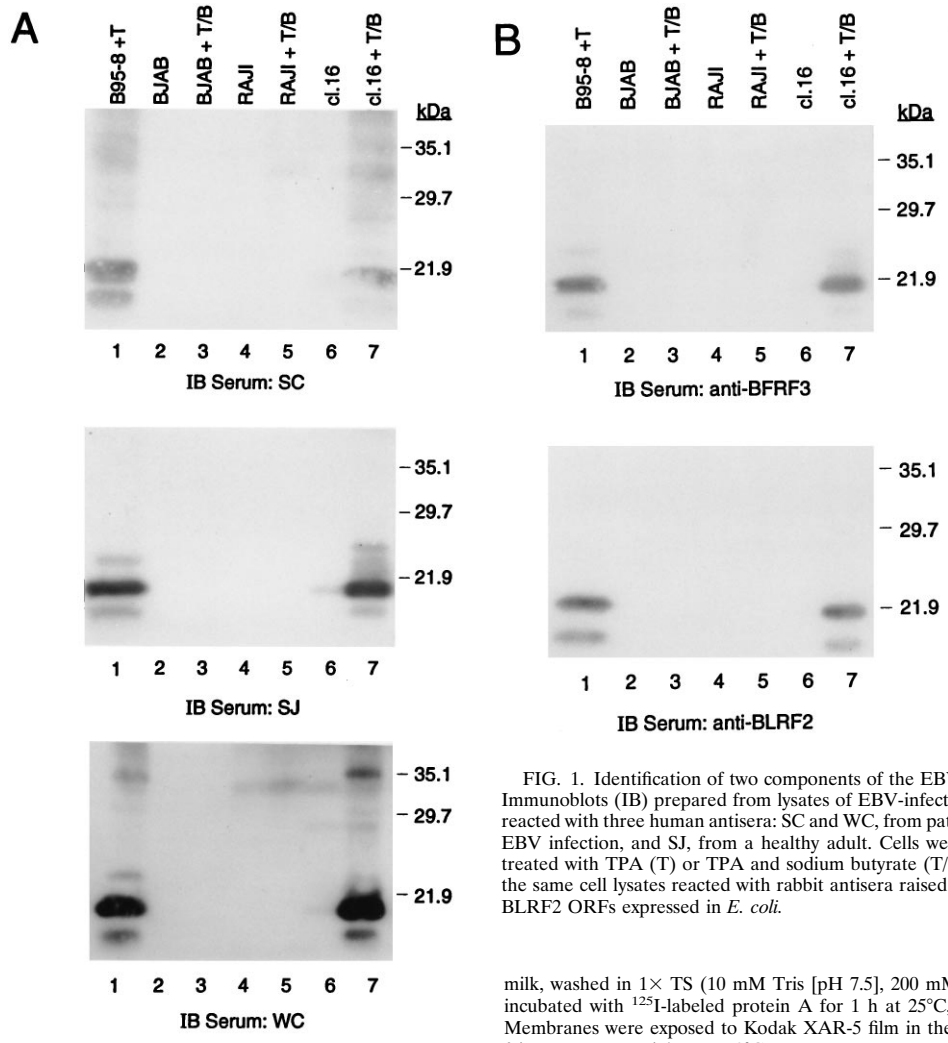


FIG. 1. Identification of two components of the EBV late p21 complex. (A) Immunoblots (IB) prepared from lysates of EBV-infected or uninfected B cells reacted with three human antisera: SC and WC, from patients with chronic active EBV infection, and SJ, from a healthy adult. Cells were either not treated or treated with TPA (T) or TPA and sodium butyrate (T/B). (B) Immunoblots of the same cell lysates reacted with rabbit antisera raised against the BFRF3 and BLRF2 ORFs expressed in *E. coli.*

ing. Cells were treated on day 3 with 10 ng of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) per ml and/or 3 mM sodium butyrate.

**Virion purification.** Supernatant fluids were harvested from B95-8 cells that had been treated with TPA and sodium butyrate for 7 days. The fluids were passed through a Millipore SSWP 3.0-μm-pore-size filter; NaCl was added to a final concentration of 2%, and one-fifth volume of 50% polyethylene glycol (PEG 8000) in 0.5 M NaCl was added. The mixture was stored overnight at 4°C and then centrifuged at 7,000 rpm for 20 min in a GSA (Beckman) rotor. The pellet was resuspended in 20 ml of phosphate-buffered saline (PBS) per liter of starting cell culture fluid. The solution was Dounce homogenized; then $MgCl_2$ was added to 10 mM, and the solution was incubated with 20 μg of DNase I per ml and 20 μg of RNase A per ml at 37°C for 4 h. EDTA was added to 50 mM. The virus was pelleted through 20% sucrose in PBS in an SW28 rotor (Beckman) at 25,000 rpm for 1 h. The pellet was resuspended in sodium dodecyl sulfate (SDS) sample buffer at 1 ml/liter of supernatant.

**Antisera.** SC and WC are human sera from patients with chronic active EBV infection (15). SJ is a human serum from a healthy EBV-positive donor. Anti-BFRF3 is a rabbit serum raised against a β-galactosidase–BFRF3 fusion protein (25). Anti-BLRF2 is a rabbit serum raised against a TrpE-BLRF2 fusion protein by methods previously described (22). ZA is a rabbit serum raised against a protein consisting of TrpE fused to amino acids 1 to 245 of BZLF1 [TrpE-BZLF1(1–245)]. C, K, and P are rabbit sera raised against a TrpE-BZLF1(1–167) fusion protein. ζ is a rabbit serum raised against a TrpE-BZLF1(197–245) fusion protein.

**Protein extracts and electrophoresis.** Cells were pelleted by centrifugation, washed with 1× PBS, and resuspended in SDS sample buffer at a concentration of $10^6$/10 μl. Extracts were heated to 100°C for 5 min and separated on SDS–10% polyacrylamide gels.

**Immunoblotting.** Separated proteins were transferred to nitrocellulose membranes at 4°C. The filters were blocked in 5% nonfat dry milk at 4°C overnight. Membranes were incubated for 1 h at 25°C with serum diluted in 5% nonfat dry milk, washed in 1× TS (10 mM Tris [pH 7.5], 200 mM NaCl, 5% Tween 20), incubated with $^{125}$I-labeled protein A for 1 h at 25°C, and washed in 1× TS. Membranes were exposed to Kodak XAR-5 film in the presence of an intensifying screen overnight at −70°C.

**Transfections.** Lymphocyte cells were resuspended in RPMI 1640 supplemented with 8% fetal calf serum at a concentration of $1.5 \times 10^7$/0.5 ml in electroporation cuvettes with a 0.4-cm gap. Ten micrograms of each expression plasmid was added to individual cuvettes. The cells were exposed to 0.25 kV and 960 μF with a Bio-Rad Gene Pulser. Growth medium was added to a total volume of 7.5 ml. LTK$^-$ cells were transfected with 10 μg of expression plasmid by the calcium phosphate method. Cells were grown at 37°C in 5% $CO_2$ for 3 days.

**Analysis of BFRF3 and BLRF2 mRNAs.** HH514-16 (clone 16) cells were subcultured in medium with or without 0.4 mM phosphonoacetic acid (PAA). The inducing agents TPA and sodium butyrate were added to half of the cells, with or without PAA, 72 h after subculture. Cytoplasmic RNA samples were prepared 24 and 48 h after induction as described previously (12). RNA representing $2.5 \times 10^6$ cells per lane was electrophoresed in a 1% agarose–6% formaldehyde gel in 20 mM MOPS (morpholinepropanesulfonic acid), pH 7. The RNA was transferred to a Nytran membrane and hybridized with a probe radiolabeled with $^{32}$P by the random priming method. The probe derived from BFRF3 was a 754-bp *Bam*HI-*Pst*I fragment (2), and the probe derived from BLRF2 was a 670-bp *Rsa*I fragment (2). The blots were reprobed with a 1.8-kb portion of β-actin cDNA.

**Expression plasmids.** The TrpE-BLRF2 expression clone was constructed by cloning the *Nsp*I (filled in by T4 DNA polymerase)-to-*Pst*I subfragment of pBR322/*Bam*HI L into pATH 10 vector which had been digested with *Sac*I (filled in by T4 DNA polymerase) and *Pst*I. pHD1013/BLRF2 was constructed by cloning the *Rsa*I subfragment of pSV2neo-*Bam*HI L (88926 to 89440 in B95-8 EBV DNA) into pHD1013 vector which had been digested with *Bam*HI and filled in by T4 DNA polymerase. pHD1013/BFRF3 was constructed by cloning the *Mlu*I-to-*Bam*HI subfragment (61501 to 62249) of the *Bam*HI F fragment of EBV into pGEM2 (Promega) which had been digested with *Hin*cII and *Bam*HI. The *Mlu*I and *Hin*cII digests were first filled in with T4 DNA polymerase. The *Pst*I 1.8-kbp fragment of the cytomegalovirus IE promoter was cloned into the *Pst*I site of this plasmid.

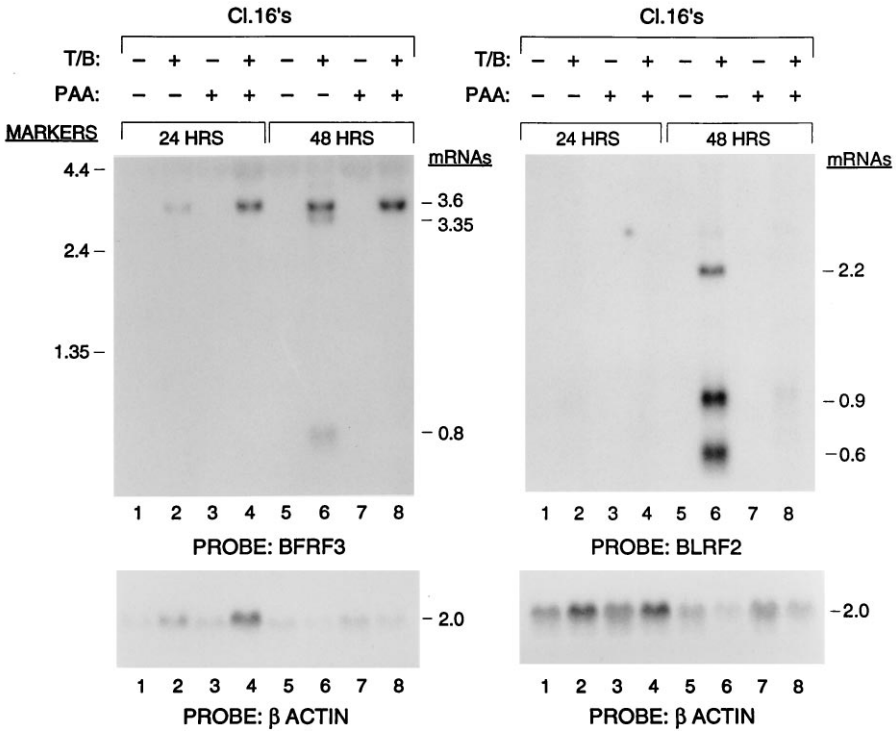


FIG. 2. BFRF3 and BLRF2 are expressed as late EBV mRNAs. Shown are Northern blots of cytoplasmic RNA prepared from HH514-16 cells 24 and 48 h after addition of inducing agents TPA and sodium butyrate (T/B). A portion of the cells had been pretreated for 72 h with PAA. The Northern blots were hybridized with portions of the BFRF3 and BLRF2 ORFs and reprobed with β-actin.

**Immunoprecipitations.** The immunoprecipitation procedure was modified from a previously described protocol (9). A total of $2 \times 10^7$ cells per immunoprecipitation were collected by centrifugation with an Eppendorf microcentrifuge (2,000 rpm, 5 min, 4°C). The cells were washed in 20 ml of cold TBS (40 mM Tris [pH 7.5], 150 mM NaCl) and centrifuged in the same manner. The pellet was resuspended in 200 μl of lysis buffer (LB) (50 mM Tris [pH 7.5], 250 mM NaCl, 0.1% Triton X-100, 5 mM EDTA, 50 mM NaF, 0.1 mM $NaVO_4$, 1 mM phenylmethylsulfonyl fluoride). The cells were sonicated three times (30 s each) at 4°C and then spun at 12,000 rpm and 4°C for 30 min. Twenty microliters of Pansorbin cells (Calbiochem) resuspended in TBS–1 mM phenylmethylsulfonyl fluoride–10% bovine serum albumin (BSA) was added to the supernatant. Preimmune serum was added, and the reaction mixture was incubated on ice for 30 min. The extract was then spun in an Eppendorf microcentrifuge at 12,000 rpm and 4°C for 15 s, and 1 μl of immune serum was added to the supernatant. The extract and serum were incubated on ice for 3 h with nutation. Twenty microliters of Pansorbin in TBS–1 mM PMSF–10% BSA was added, and the reaction mixture was incubated at 4°C overnight with nutation. The immunoprecipitates were collected by centrifugation with an Eppendorf microcentrifuge at 12,000 rpm and 4°C for 30 s. The pellet was washed with 200 μl of LB plus 0.1% SDS and resuspended in 20 μl of SDS sample buffer.

**PEPSCAN analysis.** PEPSCAN analysis was performed on antisera as previously described (24).

## RESULTS

**The p21 complex consists of late EBV-encoded products.** Human sera with EBV antibodies consistently detected a triplet of strongly immunogenic proteins in the 21-kDa range on immunoblots of B cells in which EBV had been induced into the lytic cycle (Fig. 1A). This complex was not observed in EBV-negative cells (Fig. 1A, lanes 2 and 3), in Raji cells in which lytic EBV DNA replication and late gene expression were defective (Fig. 1A, lanes 4 and 5), or in uninduced HH514-16 cells in which EBV was latent (Fig. 1A, lane 6). The viral DNA polymerase inhibitor PAA blocked production of the p21 complex in EBV-positive cell lines, such as HH514-16, that had been chemically induced into the lytic cycle (data not shown). These observations together suggested that the components of the p21 complex were encoded by EBV late genes.

**BFRF3 and BLRF2 encode two components of the p21 complex.** The EBV BFRF3 ORF has recently been shown to encode a low-molecular-weight EBV capsid protein (24). Rabbit antiserum raised against a β-galactosidase–BFRF3 fusion protein detected a triplet in the 21-kDa range with the same pattern of expression in human B cells as was seen with human antisera (Fig. 1B, top).

Immunoblots of two-dimensional electrophoresis gels on which had been run infected cell lysates, immunoprecipitates, and EBV particles showed that there were two polypeptide components of p21, both with a basic charge (8a). Computer analysis of EBV ORFs, entailing a search for late proteins of comparable molecular weight and basic charge, led to the identification of BLRF2 as a second component of p21. Rabbit antiserum specific for the BLRF2 protein was immunoreactive with the lower two bands observed in the characteristic p21 triplet (Fig. 1B, bottom). The pattern of expression of BLRF2 in cell extracts was the same as that of p21 detected with human antisera (Fig. 1A) and BFRF3 detected with rabbit antiserum (Fig. 1B, top).

Northern (RNA) blot analysis of mRNAs prepared from HH514-16 cells indicated that both BFRF3 and BLRF2 encoded true late transcripts (Fig. 2). A probe for BFRF3 detected a late 0.8-kb mRNA that was inhibited by PAA pretreatment as well as a 3.5-kb early mRNA. A probe for BLRF2 detected three late mRNAs of 2.2, 0.9, and 0.6 kb 48 h after chemical induction of HH514-16 cells; expression of all three mRNAs was inhibited by PAA.

**Reactivity of rabbit and human antisera to individually expressed BFRF3 and BLRF2 components of p21.** To analyze the reactivities of human antisera to the BFRF3 and BLRF2 com-

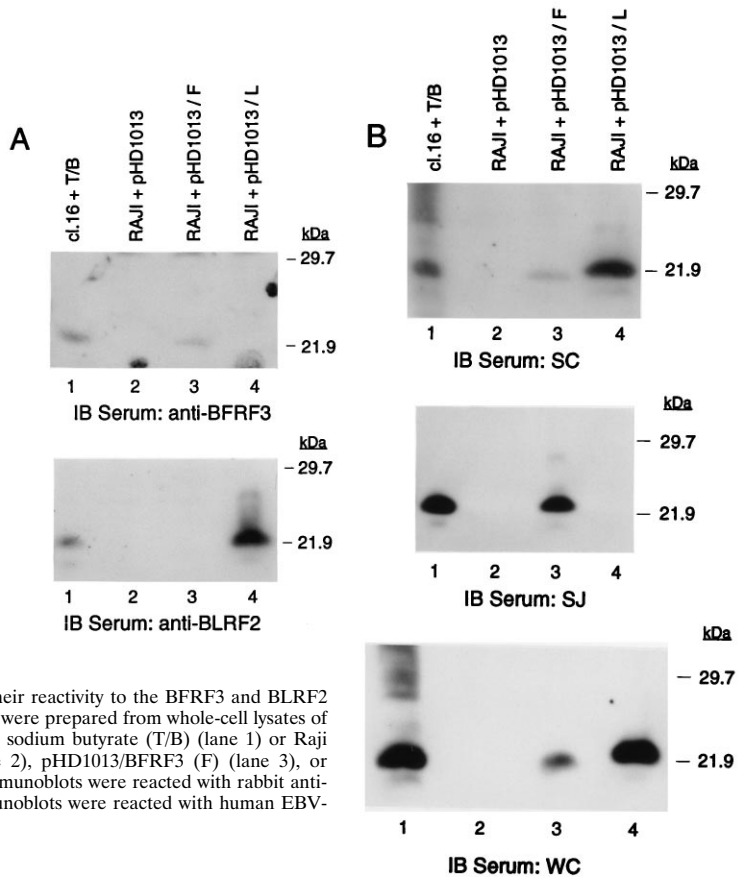


FIG. 3. Human antisera differ in their reactivity to the BFRF3 and BLRF2 components of p21. Immunoblots (IB) were prepared from whole-cell lysates of HH514-16 cells treated with TPA and sodium butyrate (T/B) (lane 1) or Raji cells transfected with pHD1013 (lane 2), pHD1013/BFRF3 (F) (lane 3), or pHD1013/BLRF2 (L) (lane 4). (A) Immunoblots were reacted with rabbit antisera to BFRF3 and BLRF2. (B) Immunoblots were reacted with human EBV-positive antisera SC, SJ, and WC.

ponents of p21, eukaryotic expression vectors for each of these ORFs were transfected into Raji cells which did not produce p21 (Fig. 1). Each product was detected with the appropriate monospecific antiserum (Fig. 3A). Sera SC and WC, from patients with chronic active EBV infection and with high titers of antibodies to EBV lytic gene products, recognized both BFRF3 and BLRF2 (Fig. 3B, top and bottom panels, lanes 3 and 4). Thus, immunoreactivity of some human sera to the p21 complex was a response to at least two gene products. Serum SJ, from a healthy adult, reacted only with BFRF3 (Fig. 3B, middle panel). Nearly all human sera with antibodies to the EBV VCA complex reacted with BFRF3 (24).

**BLRF2 was present in virions.** The other components of the VCA complex, BcLF1, BALF4, BdRF1, and BFRF3, had all been detected in viral particles (4, 13, 23). To determine if BLRF2 was also present in viral particles, immunoblots of virions which had been partially purified through sucrose gradients were analyzed. BLRF2-specific rabbit antiserum detected a 21-kDa doublet protein in virions that comigrated with intracellular p21 (Fig. 4). Whole-cell lysates of TPA-induced B95-8 cells were reactive with antisera to EBNA1 and diffuse early antigen, but the virions did not react with these antisera (data not shown). Thus, the virions did not contain appreciable amounts of nonstructural viral proteins.

**Identification of ZAP21 as BFRF3.** To investigate the relationship of ZAP21 to the two components of the p21 complex, five rabbit antisera raised against TrpE-BZLF1 fusion proteins were tested for their capacity to coimmunoprecipitate ZEBRA and p21 from B95-8 cells chemically induced into the lytic cycle (Fig. 5). While all five antisera efficiently immunoprecipitated ZEBRA, only one antiserum, ζ, coimmunoprecipitated ZEBRA with the characteristic p21 triplet (Fig. 5, lane 5). Preimmunization serum, Pζ, from the same rabbit did not immunoprecipitate p21 (Fig. 5, lane 6). ζ also coimmunoprecipitated p40 as previously described (9).

In the experiment illustrated in Fig. 5, p21 was detected with human antiserum SJ. Since SJ serum was immunoreactive with the BFRF3 protein but not with the BLRF2 protein (Fig. 3B), BFRF3 represented a strong candidate for ZAP21 that was coimmunoprecipitated by ζ antiserum. To determine if any of

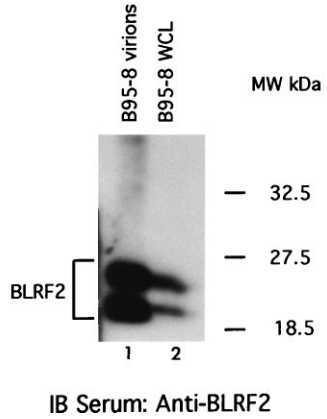


FIG. 4. Detection of BLRF2 protein in preparations of virions. Lanes: 1, virions purified from B95-8 cells by sucrose gradient centrifugation; 2, whole-cell lysate (WCL) of chemically induced B95-8 cells. An immunoblot (IB) was reacted with rabbit antiserum to BLRF2.

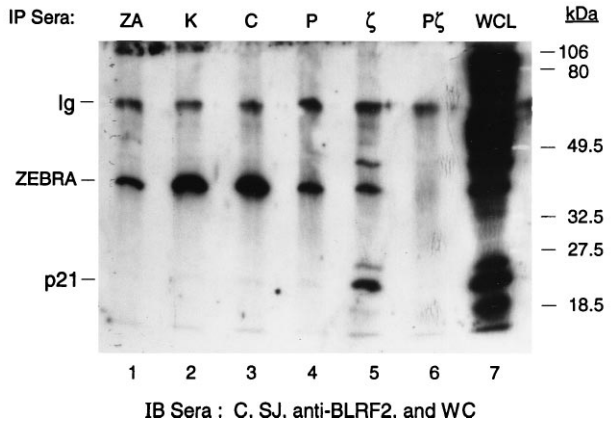


FIG. 5. Coimmunoprecipitation of p21 with rabbit antiserum ζ raised against BZLF1(197–245). Immunoprecipitations (IP) were performed with a panel of rabbit antisera to BZLF1 on whole-cell lysates of B95-8 cells treated with TPA and sodium butyrate. The rabbit antiserum ZA (lane 1) was raised against BZLF1(1–245). Rabbit antisera K (lane 2), C (lane 3), and P (lane 4) were raised against BZLF1(1–167). Rabbit antiserum ζ was raised against BZLF1(197–245). Preimmunization serum from ζ (Pζ) is found in lane 6. Whole-cell lysate (WCL) is in lane 7. The immunoprecipitation reactions and WCL were electrophoresed, immunoblotted (IB), and reacted concurrently with rabbit anti-BZLF1 (C) and human antiserum SJ. The filter was reprobed with anti-BLRF2 and subsequently with antiserum WC (see text). Ig, immunoglobulin.

the other rabbit antisera to BZLF1 were able to coimmunoprecipitate p21 encoded by BLRF2, the immunoblot illustrated in Fig. 5 was reprobed with rabbit anti-BLRF2 and with WC, a human antiserum that was also strongly reactive with BLRF2 (Fig. 3B). No additional p21 bands were seen in this portion of the experiment, indicating that none of the other rabbit antisera to BZLF1 coimmunoprecipitated a p21 protein encoded by BLRF2.

The experiments shown in Fig. 6 provided further evidence implicating BFRF3 as ZAP21. Immunoprecipitates produced by the panel of rabbit antisera to BZLF1 were immunoblotted with monospecific antisera to BFRF3 (Fig. 6A) and BLRF2 (Fig. 6C). Antiserum to BFRF3 detected p21 (Fig. 6A, lane 5); BLRF2-specific antiserum did not react with ZAP21 on an immunoblot (Fig. 6C). Reprobing the immunoblots shown in Fig. 6A with rabbit antiserum C to BZLF1 indicated that all five rabbit antisera to BZLF1 immunoprecipitated approximately the same amount of ZEBRA protein (Fig. 6B).

**Rabbit antiserum to ZEBRA(167–245) did not directly recognize BFRF3 protein on immunoblots.** To address the possibility that ζ antiserum to BZLF1 was directly reactive with BFRF3, immunoblots containing extracts of lytically induced B95-8 cells were probed with ζ antiserum as well as with P antiserum. Both antisera recognized ZEBRA on these immunoblots. Neither antiserum to BZLF1 reacted with p21. Reprobing the immunoblots with SJ indicated that BFRF3 p21 was expressed at high levels in extracts of TPA-induced B95-8 cells (data not shown).

**Rabbit antiserum ζ to ZEBRA(167–245) immunoprecipitated BFRF3 directly.** The ZEBRA-p21 coimmunoprecipitation was reconstituted in the absence of other virally encoded proteins (Fig. 7). In LTK$^-$ cells transiently expressing ZEBRA and BFRF3, ζ antiserum (Fig. 7A, lane 7), but not Pζ (lane 6) or C antiserum (lane 5), coimmunoprecipitated ZEBRA with p21-BFRF3.

During the course of these experiments, it was noted that in BFRF3-transfected EBV-negative LTK$^-$ cells (Fig. 7A, lane 10) and in BJAB cells (Fig. 7B, lane 3) the ζ antiserum was able

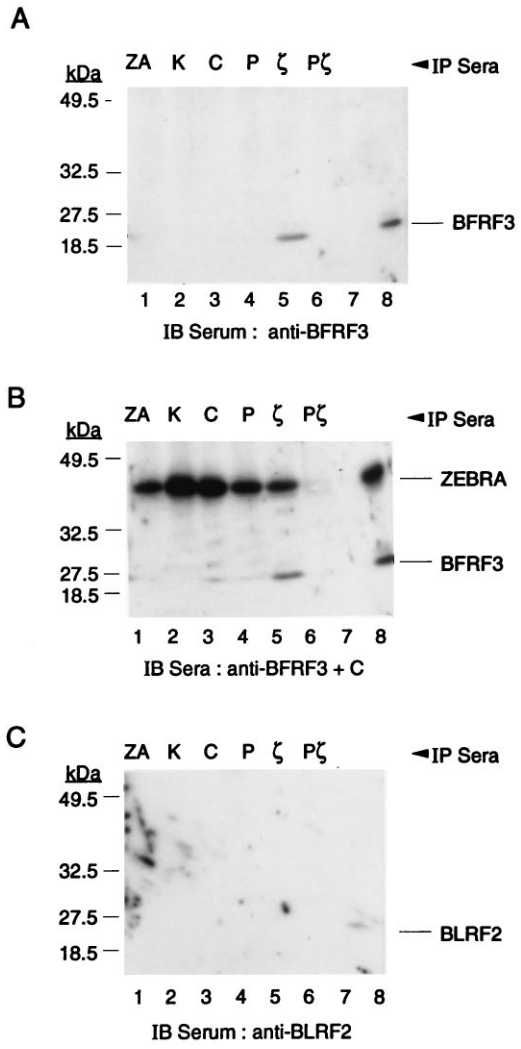


FIG. 6. Rabbit antiserum ζ to BZLF1(197–245) coimmunoprecipitates the BFRF3 component of the EBV p21 complex. Immunoprecipitations (IP) were performed on whole-cell lysates of B95-8 cells treated with TPA and sodium butyrate. The indicated immunoprecipitating rabbit antisera to BZLF1 are those described in the legend to Fig. 5. Whole-cell lysate was run in lane 8. The immunoprecipitates and whole-cell lysate were analyzed by immunoblotting (IB). (A) The immunoblotting antiserum was directed against BFRF3. (B) The immunoblot shown in panel A was reprobed with rabbit antiserum C, which was raised against ZEBRA(1–167). (C) The immunoblotting antiserum was directed against BLRF2. A longer exposure of the autoradiograph of this immunoblot failed to show a p21 band for any of the immunoprecipitates.

to immunoprecipitate BFRF3 in the absence of ZEBRA expression. The amount of BFRF3 that was immunoprecipitated with the ζ antiserum varied; this variation was related to the level of BFRF3 expressed following transfection (compare, for example, lanes 3 and 4 of Fig. 7A) (data not shown). Variation in BFRF3 expression probably also accounted for the observation that in some experiments the amount of BFRF3 immunoprecipitated was greater in the presence than in the absence of ZEBRA expression (compare lanes 7 and 10 in Fig. 7A) while in other experiments the amount of BFRF3 immunoprecipitated was greater in the absence than in the presence of ZEBRA (compare lanes 3 and 4 in Fig. 7B). From these experiments we concluded that immunoprecipitation of ZAP21 with the ζ antiserum was not dependent on expression of ZEBRA.

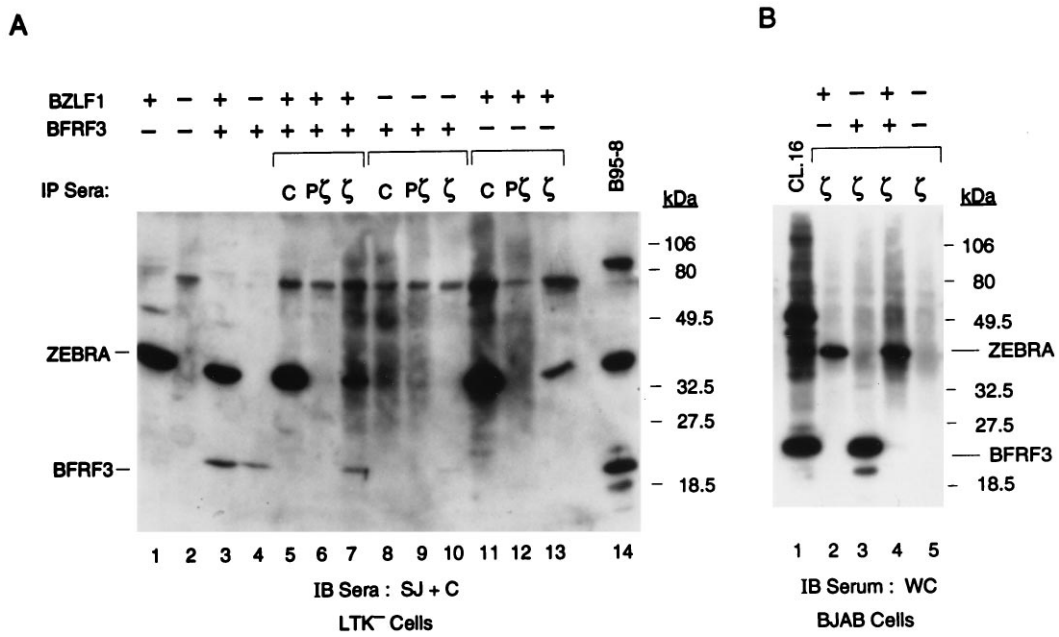


FIG. 7. ζ antiserum to ZEBRA(167–245) directly immunoprecipitates the BFRF3 component of p21. (A) Results of an experiment performed in mouse LTK⁻ cells that were transfected with cytomegalovirus expression vectors for BZLF1 and/or BFRF3. Lanes 1 to 4, whole-cell lysates; lanes 5 to 13, immunoprecipitations (IP) with rabbit antisera C, Pζ, or ζ. The whole-cell lysates and immunoprecipitates were analyzed by immunoblotting (IB) concurrently with human antiserum SJ to BFRF3 p21 and with rabbit antiserum C to ZEBRA. (B) Results of an experiment performed in BJAB cells that were transfected with cytomegalovirus expression vectors for BZLF1 and/or BFRF3. Whole-cell lysates of HH514-16 cells or immunoprecipitates of transfected BJAB cells were analyzed by immunoblotting with human antiserum WC, which recognizes BFRF3 and BLRF2 p21.

**Direct recognition of an epitope of BFRF3 by the ζ antiserum.** The ζ antiserum could immunoprecipitate BFRF3 in the absence of ZEBRA by direct recognition of BFRF3 or by indirect recognition of a cellular protein that interacts with BFRF3-p21. To investigate whether the ζ antiserum reacted with p21-BFRF3 directly or indirectly, a series of overlapping 12-amino-acid-residue peptides of BFRF3 were incubated with ζ antiserum and the reactions were quantitated by enzyme-linked immunosorbent assay (Fig. 8A). The ζ antiserum reacted with peptides spanning aa 147 to 167 of the BFRF3 ORF. The P antiserum to BZLF1, which did not immunoprecipitate ZAP21 (Fig. 4, 5, and 7), did not react with the BFRF3 peptides by PEPSCAN (Fig. 8B). Rabbit antiserum raised to a β-galactosidase–BFRF3 fusion recognized the same region of BFRF3 as did the ζ antiserum to BZLF1 (Fig. 8C). However, a rat monoclonal antibody to BFRF3 recognized a different portion of the BFRF3 protein (Fig. 8D).

## DISCUSSION

This report identifies a second EBV gene, BLRF2, that encodes a component of the viral late p21 complex. Thus, EBV p21 consists of two gene products, BFRF3 and BLRF2. We show that BFRF3 is the previously described ZEBRA-associated protein p21.

**Two components of p21.** BFRF3 and BLRF2 share many characteristics. Both proteins are present in extracts of cells induced into late lytic cycle gene expression and are absent in extracts of cells in which lytic replication is arrested during early gene expression (Fig. 1B and data not shown). Both proteins are basic and are expressed from late transcripts (2, 3, 25) (Fig. 2). BFRF3 was previously misclassified as an early gene (7). Inhibition of expression of the 0.8-kb true late BFRF3 transcript requires that the cells be pretreated with the viral DNA polymerase inhibitor PAA prior to induction of the lytic cycle (25).

The products of BFRF3 and BLRF2 are both recognized by human antisera with antibodies to VCA (Fig. 3). Antibodies to BFRF3 correlate with the presence of antibodies to VCA in late-convalescent-phase sera (24). However, antibodies to BFRF3 arise relatively slowly after primary infection, whereas antibodies to the VCA complex as a whole are present early after primary infection (20). Further work is needed to describe the frequency and kinetics of the antibody response to BLRF2. A recent report suggests that many EBV-positive human antisera recognize an antigen encoded by BLRF2 (19). However, work presented here (Fig. 3) indicates that certain anti-VCA-positive sera, such as SJ, lack detectable reactivity with BLRF2. BFRF3, together with other characterized components of the VCA complex, such as BcLF1, BALF4, and BdRF1, is detected in preparations of viral particles (4, 13, 25). The BLRF2-specific antiserum also detected a 21-kDa doublet in virions purified from extracellular fluids of the B95-8 cell line by sucrose gradient centrifugation (Fig. 4). Thus, both p21 proteins are likely to be structural components of EB virions. BFRF3 has homology with capsid proteins of herpesvirus saimiri (ORF 65) (1) and human herpesvirus 8 (20a). BLRF2 has homology to herpesvirus saimiri genes 52 and 64, the latter being a probable large tegument protein. It is interesting that BFRF3 is homologous to ORF 65 in herpesvirus saimiri.

Antisera raised specifically to each of the two ORFs detected multiple distinct bands in infected-cell extracts and in preparations of viral particles (Fig. 1 and 3). The appearance of distinct bands suggests that posttranslationally modified forms of both proteins are present in infected cells and in virions at the steady-state level. These different forms may alter the function of the two proteins intracellularly in the assembly of virions or in infectivity.

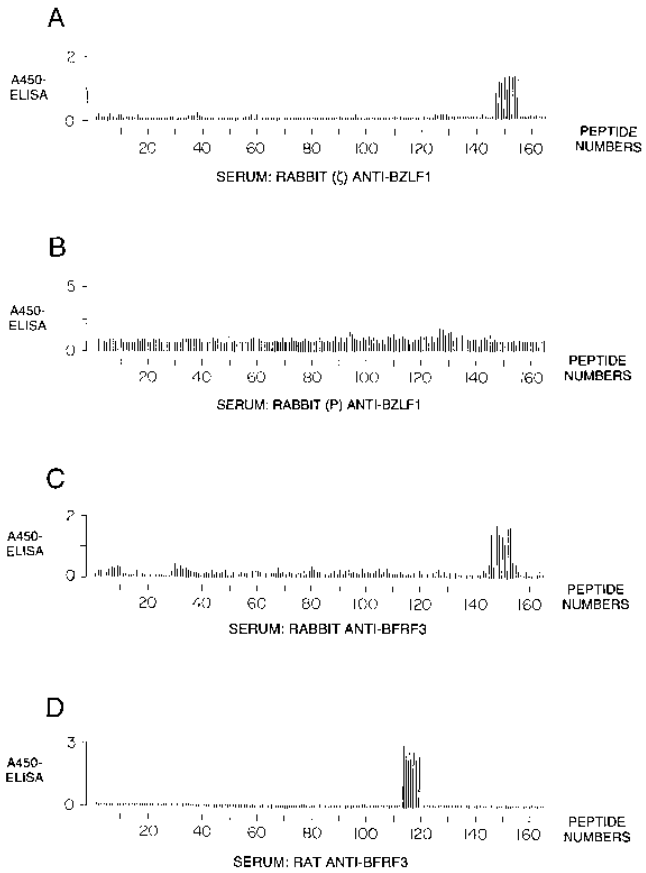


FIG. 8. PEPSCAN analysis with overlapping peptides of the BFRF3 protein. The peptides of BFRF3 were reacted with rabbit antisera to BZLF1, ζ (A) and P (B), or antiserum to BFRF3 made in rabbits (C) or rats (D).

**BFRF3 is ZAP21.** The availability of monospecific antisera to BFRF3 and BLRF2 and the construction of eukaryotic expression vectors for each of the two ORFs allowed us to demonstrate that BFRF3 was the previously described ZAP21. Monospecific antiserum to BFRF3, but not antiserum to BLRF2, reacted with ZAP21 coimmunoprecipitated with ZEBRA from EBV-positive cell lines induced into the late lytic cycle (Fig. 6). Reconstruction experiments in EBV-negative cells (BJAB and LTK$^-$), as well as in Raji cells transfected with expression vectors for BFRF3 and BLRF2, indicated that BFRF3, but not BLRF2, was coimmunoprecipitated with ZEBRA (Fig. 7 and data not shown).

Of the five antisera to BZLF1, only antiserum ζ reproducibly coimmunoprecipitated ZAP21 (Fig. 5 and 6). Katz et al. reported that antiserum P was also able to coimmunoprecipitate ZAP21 from EBV-infected cells induced into the late lytic cycle (9). However, changes in the immunoprecipitation protocol, i.e., a longer preclearing centrifugation step following cell lysis, led to the disappearance of this activity.

The failure of some ZEBRA antisera to coimmunoprecipitate ZAP21 was previously explained by an epitope interference hypothesis. It was postulated that some antibodies which react with ZEBRA could interfere with the association with ZAP21. However, the current experiments provide another explanation for the differences among antisera: ζ antiserum, but not P antiserum, recognizes an epitope on the BFRF3 protein itself (Fig. 8).

The cross-reactivity of the ζ antiserum is likely to be conformational, since it does not recognize p21-BFRF3 on immunoblots (data not shown) even though it can immunoprecipitate BFRF3 (Fig. 7) and react with overlapping peptides near the carboxy terminus of BFRF3 (Fig. 8). The ζ antiserum recognized two overlapping peptides of the predicted BFRF3 sequence. These peptides shared the epitope TGSG, corresponding to amino acids 156 to 159 of BFRF3. This sequence, however, was not present in the predicted BZLF1 sequence. Therefore, cross-reactivity may have been due to a conformational rather than a linear epitope shared by BZLF1 and BFRF3, as suggested by the failure of ζ to detect BFRF3 by Western blotting (immunoblotting). Alternatively, the bacterially expressed ZEBRA(197–245) protein immunization extract may have contained an *Escherichia coli* protein that shares antigenicity with BFRF3(147–167).

An interesting hypothesis raised by the original observation that ZEBRA was coimmunoprecipitated with p21, an EBV capsid protein, was that ZEBRA itself was associated with virions (9). Certain forms of ZEBRA present in virions might be important in initiation of the lytic cycle in epithelial cells or in repression of the lytic cycle in lymphocytes. Although our findings make it less likely that ZEBRA is directly associated with BFRF3, BLRF2, or other late proteins, they do not exclude the original hypothesis that ZEBRA is packaged into virions. Preliminary experiments suggest that certain modified forms of ZEBRA are selectively present in virion preparations (20a).

## ACKNOWLEDGMENTS

This work was supported by grants from the NIH (AI22959 and CA16038) to G.M. and a Howard Hughes Medical Institute predoctoral fellowship to T.R.S.

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