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The Epstein-Barr Virus (EBV) BZLF2 Gene Product Associates with the gH and gL Homologs of EBV and Carries an Epitope Critical to Infection of B Cells but Not of Epithelial Cells

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Glycoprotein gp85, the product of the BXLF2 open reading frame (ORF), is the gH homolog of Epstein-Barr virus (EBV) and has been implicated in penetration of virus into B cells. Like its counterparts in other herpesviruses, it associates with a gL homolog, gp25, which is the product of the BKRF2 ORF. Unlike the gH homologs of other herpesviruses, however, gp85 also complexes with two additional glycoproteins of 42 and 38 kDa. Glycoproteins gp42 and gp38 were determined to be alternatively processed forms of the BZLF2 gene product. Coexpression of EBV gH and gL facilitated transport of gH to the cell surface and resulted in formation of a stable complex of gH and gL. It also restored expression of an epitope recognized by monoclonal antibody E1D1, which immunoprecipitates the native gH complex but not recombinant gH expressed in isolation. Coexpression of gH, gL, and the BZLF2 ORF restored expression of an epitope recognized by a second monoclonal antibody, F-2-1, which immunoprecipitates the native gH-gL-gp42/38 complex but not the complex of recombinant gH and gL alone. The epitope recognized by antibody F-2-1 was mapped to the BZLF2 gene product itself. Antibody F-2-1 inhibited the ability of EBV to infect B lymphocytes but had no effect on the ability of the virus to infect the epithelial cell line SVK-CR2. In contrast, antibody E1D1 had no effect on infection of the B-cell line but inhibited infection of the epithelial cell line. These results indicate that penetration of the two cell types by EBV involves differential use of the gH-gL-gp42/38 complex and suggest the hypothesis that the BZLF2 gene product has evolved as a unique adaptation to infection of B lymphocytes by EBV.

Entry of herpesviruses into cells is a complex event that probably requires the activities of several unique glycoproteins. However, despite the differences in host range and tissue tropism of the members of this large family, some common themes in the process have been emerging. Among these are the involvement of the herpes simplex virus gH and its homologs in virus penetration (4–7, 14, 18) and the requirement for gH homologs to associate with a second conserved glycoprotein, gL, in order to be correctly folded and transported within the infected cell (3, 9, 10, 12, 20, 21). The Epstein-Barr virus (EBV) gH homolog is gp85, the product of the BXLF2 open reading frame (ORF) (8, 17), and in many respects this glycoprotein mirrors the behavior of its counterparts in other viruses. Two monoclonal antibodies, F-2-1 and E1D1, have been used to characterize the molecule biochemically and implicate the EBV gH as playing a role in virus penetration. One of the two antibodies, F-2-1, has been shown to neutralize infection of B cells; the other, E1D1, is nonneutralizing for infection of this cell type. Monoclonal antibody F-2-1 has no effect on virus binding to B lymphocytes but, as judged by a fluorescence dequenching assay, inhibits virus fusion with the cell membrane (14). Virosomes made from EBV proteins bind and fuse with receptor-positive B cells unless the proteins are depleted of gH by affinity chromatography with antibody E1D1, in which case they still bind but fail to fuse (7). If gH is expressed in isolation as a recombinant protein, it carries no sugars that are resistant to digestion with endoglycosidase H, it is not transported to the cell surface, and it is no longer immunoprecipitated by either of the two monoclonal antibodies F-2-1 and E1D1. The native protein coprecipitates with a glycoprotein of 25,000 Da (gp25) which is recognized by an antipeptide antibody made to a sequence derived from the predicted BKRF2 ORF, a positional homolog to the herpes simplex virus UL1 gene that encodes gL (25).

There are also, however, some interesting and perhaps functionally important differences between the EBV gH-gL complex and those of other herpesviruses. Antibodies F-2-1 and E1D1 immunoprecipitate not only gH and gL but also a third glycoprotein of 42,000 Da (gp42) of previously unknown origin (25). We report here that gp42 is the product of the BZLF2 ORF. This ORF is predicted to encode a 223-amino-acid protein (1) with a potential signal peptide but no other hydrophobic region predicted to be membrane spanning. It has no apparent homologs in other known human herpesviruses. Coexpression of gH with gL proved to be sufficient for transport of gH to the cell surface and for restoration of reactivity with monoclonal antibody E1D1. In contrast, however, immunoprecipitation of the entire complex by antibody F-2-1 required coexpression of gp42, since this is the member of the complex that carries the epitope recognized by the antibody. Expression of gp42 in the cell membrane did not require coexpression of any other EBV protein and suggested that the glycoprotein is anchored by an uncleaved signal peptide.

Although EBV has long been known to infect at least two cell types, B lymphocytes and epithelial cells, in vivo, an in vitro model for infection of epithelial cells has only recently become available. Li and colleagues derived a simian virus 40-transformed cell line (SVK-CR2) that was stably transfected with

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the B-cell receptor for EBV, CR2, which binds with high affinity to the EBV attachment protein gp350/220 (11). These cells are particularly susceptible to infection with the Akata strain of EBV. We report here that the two antibodies to the gH complex, E1D1 and F-2-1, have reciprocal effects on infection of SVK-CR2 cells and B cells by Akata virus. F-2-1 neutralized infection of B cells but had no effect on infection of the SVK-CR2 line; E1D1 neutralized infection of SVK-CR2 cells but had no effect on infection of lymphocytes. Collectively, these results suggest that EBV has added a unique glycoprotein to its gH-gL complex that may be essential for infection of the B cell and that the tripartite gH-gL-gp42 complex interacts differently with lymphocytes and epithelial cells.

MATERIALS AND METHODS

Cells. Akata, a Burkitt lymphoma-derived cell line that can be induced to make EBV (23) (a gift of John Sixbey, St. Jude Childrens' Research Hospital, Memphis, Tenn.), and MCUV5, a marmoset cell line that spontaneously produces EBV (a gift of George Miller, Yale University, New Haven, Conn.), were grown in RPMI 1640 (Sigma Chemical Co., St. Louis, Mo.) supplemented with 10% (Akata) or 5% (MCUV5) heat-inactivated fetal bovine serum (Gibco/BRL Life Technologies, Grand Island, N.Y.). CV-1 cells were grown in Dulbecco modified Eagle's medium containing 5% heat-inactivated fetal bovine serum. SVK-CR2 cells (11) (a gift of A. B. Rickinson, University of Birmingham, Birmingham, England) were grown in Joklik's modified Dulbecco modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan Utah) and 10 ng of cholera toxin (Sigma) per ml. Human leukocytes were obtained from heparinized adult peripheral blood or from cord blood by flotation on lymphocyte separation medium (Organon Teknika, West Chester, Pa.). Adult leukocytes were depleted of T cells by a double cycle of rosetting with 2-aminoethylisothiouronium bromide-treated sheep erythrocytes (19) and centrifugation on 60% Percoll (Pharmacia Fine Chemicals, Piscataway, N.J.).

Virus. Stocks of recombinant vaccinia virus expressing the T7 RNA polymerase (vvT7 [15]) (a gift of William Britt, University of Alabama, Birmingham) were grown in CV-1 cells infected at a multiplicity of infection of 0.01 and harvested by freeze-thawing and sonication of cells. EBV to be used for protein analysis was derived from concentrated culture medium of induced Akata cells as previously described (25). Virus to be used for cell infection was harvested from unconcentrated clarified culture medium that had been passed through a 1.2-μm-pore-size filter.

Plasmid constructs. The BXLF2, BKRF2, and BZLF2 ORFs were amplified by a PCR method from EBV DNA isolated by digestion of MCUV5 cells with proteinase K at 65°C for 2 h in 0.5% sodium dodecyl sulfate (SDS)–50 mM EDTA (pH 8.0) followed by phenol-chloroform extraction. Each DNA fragment was cloned into plasmid pTM1 (15) (a gift of Radha Padmanabhan, Kansas University Medical Center, Kansas City), which contains the T7 promoter, the encephalomyocarditis virus cap-independent translation signal, a multiple cloning site, and a T7 transcriptional terminator. Translation initiates at the *Nco*I site in the multiple cloning region. The BXLF2 5′ primer (CAT TTG CTC TGT GTT TTT TGC C) included the sequences immediately following the first ATG of the ORF. The 3′ primer (GGC GC↓T CGA GCC TGC TGC GCA GCA TCT A) included the stop codon, 3′ untranslated sequences, and an *Xho*I site. The DNA amplified with these primers was cut with *Xho*I and inserted into pTM1 that had previously been cut with *Nco*I, blunt ended, and recut with *Xho*I to make plasmid pTM1-gH. The BKRF2 5′ primer (CGT GCT GTT GGT GTA TTT CTG GCC ATC TGT C) included the sequences immediately following the first ATG of the ORF. The 3′ primer (GGC G↓G ATC CCT TCC GGC AGA GGT CTA GC) included the BKRF2 stop codon, 3′ untranslated sequences, and a *Bam*HI site. The DNA amplified with these primers was cut with *Bam*HI and cloned into pTM1 that had previously been cut with *Nco*I, blunt ended, and recut with *Bam*HI to make plasmid pTM1-gL. The BZLF2 5′ primer (GGT TGC↓CAT GGT TTC ATT) included the first ATG of the ORF and an *Nco*I site. The 3′ primer (GGC GGA GCT↓ CGC TTA GCT ATT TGA TCT) included the BZLF2 stop codon and a *Sac*I site. The DNA amplified by these primers was cut with *Nco*I and *Sac*I and cloned into pTM1 that had previously been cut with the same enzymes to make plasmid pTM1-BZLF2.

Transfection-infection protocol. CV-1 cells were grown to 90% confluency in 100-mm-diameter petri dishes and infected with vvT7 at a multiplicity of infection of 5. Thirty minutes later, the inoculum was removed, and the cells were washed twice in medium without serum and transfected with one, two, or three plasmids. For transfection of a single plasmid, 7 μg of DNA was mixed with 20 μl of Lipofectin (Gibco/BRL) made to a total volume of 200 μl with serum-free medium. Transfection of two plasmids used 14 μg of DNA and 30 μl of Lipofectin made to 400 μl in medium; transfection of three plasmids used 21 μg of DNA and 50 μl of Lipofectin made to 400 μl with medium. Each mixture was incubated for 30 min at room temperature before addition to cells. Six hours later, the total volume of medium was made up to 4 ml, and serum was added to a final concentration of 2%. Transfected cells were harvested after an additional 16 h of incubation.

Antibodies. Monoclonal antibodies F-2-1 (22) and E1D1 (17) were obtained from spent culture medium of hybridoma cells. Three antipeptide antibodies were made to synthetic peptides corresponding to residues 518 to 533 of the BXLF2 ORF (anti-gH), to residues 125 to 137 of the BKRF2 ORF (anti-gL), and to residues 71 to 88 of the BZLF2 ORF (anti-BZLF2). For ease of coupling to keyhole limpet hemocyanin according to the procedure of Liu and colleagues (13), a cysteine residue was added to the amino terminus of each peptide. Rabbits were immunized as previously described (25). All antibodies were purified by chromatography on protein A (Sigma) coupled to Affigel-15 (Bio-Rad, Richmond, Calif.), and antipeptide antibody anti-gH was further purified by affinity chromatography on peptide coupled to Affigel-10.

Immunofluorescence. Immunofluorescence staining of cytoplasmic proteins was done as previously described (25). For anticomplement immunofluorescence staining for EBNA, cells were air dried on slides, fixed for 10 min in ice-cold acetone-methanol (1:1), and reacted sequentially for 1 h each time with EBV-seropositive or -seronegative human serum, seronegative human serum as a source of complement, and fluorescein-conjugated goat anti-human C3 (Organon Teknika). For staining of virus proteins on the cell surface, cells were reacted with antibody in suspension and were fixed briefly in 0.1% paraformaldehyde in phosphate-buffered saline (pH 7.2) at the end of the procedure.

Virus neutralization. The effect of antibody on transformation of B cells with virus was tested by incubating 100 μl of Akata virus with an equal volume of antibody at a concentration of 100 μg/ml for 1.5 h at 37°C. Virus and antibody were then incubated for an additional 2 h with 5×10^5 T-cell-depleted human peripheral leukocytes. The volume of the cell suspension was increased to 1.5 ml with RPMI 1640 supplemented with 10% heat-inactivated serum, and cells were plated in quintuplicate in 96-well round-bottom tissue culture plates at a concentration of 1×10^5 to 2×10^5 cells per well. Cells were fed once a week by replacing half of the medium, and they were examined for evidence of transformation after 5 weeks. Neutralization was also determined by measuring the amount of human antibody made by T-cell-depleted peripheral leukocytes after 12 days in culture with virus as previously described (14). The effect of antibody on infection of SVK-CR2 cells was examined by incubating 250 μl of Akata virus with an equal volume of antibody at a concentration of 100 μg/ml for 1.5 h at 37°C. Antibody and virus were then added to duplicate cultures of cells grown to 80 to 90% confluency in 24-well tissue culture plates, and 500 μl of Joklik's medium was added to cover the cells. An additional 1 ml of medium was added 3 to 4 h later, and cells were reincubated for 4 days. Cells were removed from dishes by trypsinization, allowed to recover in medium with serum for 30 min at 37°C, washed in phosphate-buffered saline, air dried on slides, and stained for EBNA as described above or processed for Western immunoblotting.

Radiolabeling and immunoprecipitation. EBV was labeled extrinsically with ^{125}I (Amersham Corp., Arlington Heights, Ill.) after pelleting from 4 ml of concentrated culture supernatant or labeled biosynthetically with [^{35}S]cysteine (1,300 Ci/mmol; Amersham) or [^{3}H]glucosamine (20 Ci/mmol; Amersham) for 20 h at 6 h after induction with anti-human immunoglobulin G as previously described (25). CV-1 cells that had been infected with recombinant vaccinia virus and transfected with plasmid DNA were labeled biosynthetically with 400 μCi of [^{35}S]cysteine, with 100 μCi of Pro-Mix (70% [^{35}S]methionine, 30% [^{35}S]cysteine [>1,000 Ci/mmol, Amersham]) and 300 μCi of [^{35}S]cysteine, or with 400 μCi of [^{3}H]glucosamine per dish (approximately 10^7 cells) 4 h after transfection in 2 ml of medium containing 1/10 normal concentration of cysteine, cysteine and methionine, or glucosamine. Two hours later an additional 2 ml of medium was added, the serum concentration was adjusted to 2% with heat-inactivated dialyzed fetal bovine serum, and the cells were reincubated for an additional 16 h. CV-1 cells were labeled extrinsically with ^{125}I 18 to 20 h posttransfection. Cells were scraped from the dishes, washed three times in phosphate-buffered saline, resuspended in 500 μl of phosphate-buffered saline, and labeled with 1 mCi of ^{125}I by use of Iodobeads. Labeled cells and virus were solubilized in radioimmunoprecipitation buffer (50 mM Tris-HCl, [pH 7.2], 0.15 M NaCl, 1% sodium deoxycholate, 0.1% SDS, 0.1 mM phenylmethylsulfonyl fluoride, 100 U of aprotinin per ml) and immunoprecipitated with antibody and protein A-Sepharose CL4B (Sigma). Immunoprecipitated proteins were washed, dissociated by boiling in sample buffer containing 2-mercaptoethanol, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) in acrylamide cross-linked with 0.28% *N,N′*-diallyltartardiamide followed by fluorography.

Oligosaccharide digestion. For removal of sugars, immunoprecipitated proteins were washed in radioimmunoprecipitation buffer and resuspended in buffer containing 50 mM EDTA, 100 mM sodium phosphate buffer (pH 7.2), 0.1% SDS, 1% 2-mercaptoethanol, and 0.5% *n*-octylglucoside, boiled for 3 min, cooled, and incubated at 37°C for 20 h with the addition of either 2 U of endoglycosidase F/N or 5 mU of neuraminidase and 2.5 U of *O*-glycanase (Boehringer Mannheim). Digested samples were analyzed by SDS-PAGE and fluorography.

Sucrose gradient sedimentation. Ten million induced Akata cells were lysed in 1 ml of radioimmunoprecipitation buffer and layered on a 9-ml gradient of 5 to 25% sucrose over a 60% sucrose cushion. Sucrose was made up in buffer containing 50 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 0.1% Triton X-100, 0.5% deoxycholate 0.1 mM phenylmethylsulfonyl fluoride, and 100 U of aprotinin per ml. The gradients were centrifuged for 18 h at 38,000 rpm in an SW41 rotor

(180,000 × *g*, average) at 20°C. One-milliliter fractions were collected and immunoprecipitated before analysis by SDS-PAGE and fluorography.

Western blotting. Cells were lysed in immunoprecipitation buffer, electrophoresed in polyacrylamide, and then electrically transferred onto nitrocellulose membranes (0.45-μm pore size; Schleicher & Schuell, Inc., Keene, N.H.) at 20 mA for 18 h. The transferred sheets were treated for at least 3 h with blocking buffer (10 mM Tris-HCl [pH 7.2], 0.15 M NaCl, 5% skim milk, 0.05% sodium azide) and reacted for at least 3 h with blocking buffer containing rabbit antibody at 100 μg/ml. They were then washed five times with wash buffer (10 mM Tris-HCl [pH 7.2], 0.15% NaCl, 0.3% Tween 20) for 10 min each time and then subjected an overnight wash. The washed sheets were reacted with alkaline phosphatase-conjugated goat anti-rabbit antibodies (HyClone) for 3 h, and the bound anti-rabbit antibodies were detected by reacting with substrate bromo-4-chloroindophosphate and Nitro Blue Tetrazolium (Sigma).

In vitro transcription and translation. Plasmid pTM1-BZLF2 was linearized with *Xho*I, and mRNA was transcribed by using a MEGAscript kit (Ambion Inc., Austin, Tex.). The uncapped RNA was translated by using a rabbit reticulocyte lysate system (Ambion) and [^{35}S]cysteine (Amersham) to label protein. Protein was analyzed by SDS-PAGE and fluorography.

RESULTS

Coexpression of gp85-gH and gp25-gL. The BKRF2 ORF is predicted to encode a 137-amino-acid protein which has a potential signal peptide and no other hydrophobic sequence long enough to be membrane spanning. It includes three potential N-linked glycosylation sites. We previously showed that the anti-peptide antibody anti-gL made to sequences corresponding to the 13 carboxy-terminal residues of the predicted BKRF2 sequence immunoprecipitated the gH complex by virtue of its reactivity with gL (25). However, without expression of both gH and gL in isolation from other EBV proteins, we could not determine whether coexpression produced a stable complex of proteins, whether the two were sufficient for transportation of gH to the cell surface, or whether reactivity with monoclonal antibodies F-2-1 and E1D1 could be restored. The gH and gL ORFs were therefore cloned into plasmid pTM1 for expression under control of the T7 promoter. These plasmids were transfected into CV-1 cells that were also infected with recombinant vaccinia virus expressing the T7 polymerase. Proteins of the appropriate sizes that reacted in Western blots with antipeptide antibodies anti-gH and anti-gL were made in cells transfected with either the plasmid expressing the BXLF2 ORF (pTM1-gH), the BKRF2 ORF (pTM1-gL), or both (Fig. 1A); as previously demonstrated in immunoprecipitation assays (25), there was no cross-reactivity between the two antibodies. Cotransfection of pTM1-gH and pTM1-gL then reconstituted a complex of gH and gL that could be immunoprecipitated from [^{35}S]cysteine-labeled cells by the antipeptide antibody anti-gL (Fig. 1B). To verify that coexpression of the two proteins also resulted in transport of gH to the cell surface, cells were transfected with either one or both plasmids and labeled extrinsically with ^{125}I. Both antibody anti-gH, which reacts primarily with uncomplexed gH in EBV-producing cells (data not shown), and anti-gL, which reacts with complexed or uncomplexed gL, immunoprecipitated labeled gH from cells that were transfected with the two plasmids but not from cells transfected with either one alone (Fig. 2). In contrast, the amounts of labeled gL that were immunoprecipitated from cells that were singly transfected with pTM1-gL or doubly transfected were equivalent, suggesting that coexpression might not be required for cell surface transport of this protein. Indirect immunofluorescence assays revealed that coexpression of gH and gL had reconstituted the epitope recognized by monoclonal antibody E1D1 (Fig. 3). However, the epitope recognized by antibody F-2-1 was not detected in cells expressing gH alone, gL alone, or the two together.

Coexpression of gH, gL, and gp42. The failure of coexpression of gH and gL to restore recognition of the complex by antibody F-2-1 suggested that the third glycoprotein associated

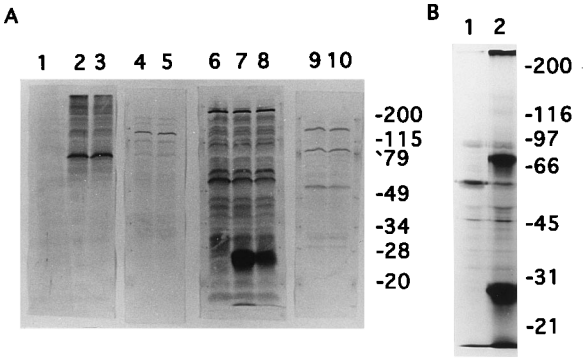

FIG. 1. (A) SDS-PAGE analysis and Western blot of CV-1 cells transfected with plasmid pTM1 (lanes 1, 4, 6, and 9), pTM1-gH (lane 2), pTM1-gL (lane 7), or pTM1-gH and pTM1-gL (lanes 3, 5, 8, and 10) and infected with recombinant vaccinia virus expressing the T7 polymerase. Blots were reacted with antipeptide antibody anti-gH (lanes 1 to 3), preimmune antibody from the same rabbit (lanes 4 and 5), antipeptide antibody anti-gL (lanes 6 to 8), or preimmune antibody from the same rabbit (lanes 9 and 10). (B) SDS-PAGE analysis of proteins immunoprecipitated by antipeptide antibody anti-gL from CV-1 cells labeled for 18 h with [^{35}S]cysteine 4 h after transfection with plasmid pTM1 (lane 1), or with plasmids pTM1-gH and pTM1-gL (lane 2), and infection with recombinant vaccinia virus expressing T7 polymerase. Sizes are indicated in kilodaltons.

with gH in virus might be important to reconstitution of the epitope. This third member of the complex, which could be immunoprecipitated from EBV by antibody E1D1, F-2-1, or anti-gL, could frequently be resolved into two species of 42,000 and 38,000 Da (Fig. 4B). Like gL, which lacks methionine residues, neither could be labeled with [^{35}S]methionine (data not shown). To determine the sizes of the proteins in the absence of sugar residues, the gH complex was immunoprecipitated from ^{125}I-labeled virus or [^{35}S]cysteine-labeled virus-producing cells and digested with endoglycosidase F/N or neuraminidase and *O*-glycanase. Neuraminidase and *O*-glycanase had no effect on the mobility of the proteins, but endoglycosidase F/N, which reduced gL to a predominant species of approximately 15,000 Da (its predicted mass without sugar), reduced the 42,000- and 38,000-Da species to two molecules of 32,000 and 28,000 Da that migrated above and below, respectively, the 31,000-Da molecular weight marker (Fig. 4). A search was then made of the predicted ORFs of the EBV genome for a protein(s) with a mass of 28,000 to 32,000 Da,

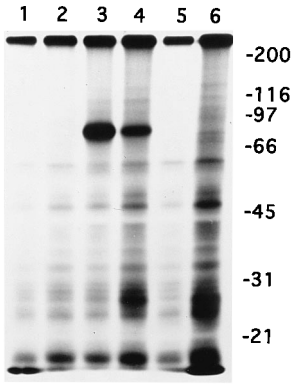

FIG. 2. SDS-PAGE analysis of proteins immunoprecipitated by antipeptide antibody anti-gH (lanes 1, 3, and 5) or anti-gL (lanes 2, 4, and 6) from CV-1 cells labeled with ^{125}I at 18 h posttransfection. Cells were transfected with plasmid pTM1-gH alone (lanes 1 and 2), plasmids pTM1-gH and pTM1-gL (lanes 3 and 4), or plasmid pTM1-gL alone (lanes 5 and 6) and infected with recombinant vaccinia virus expressing T7 polymerase. Sizes are indicated in kilodaltons.

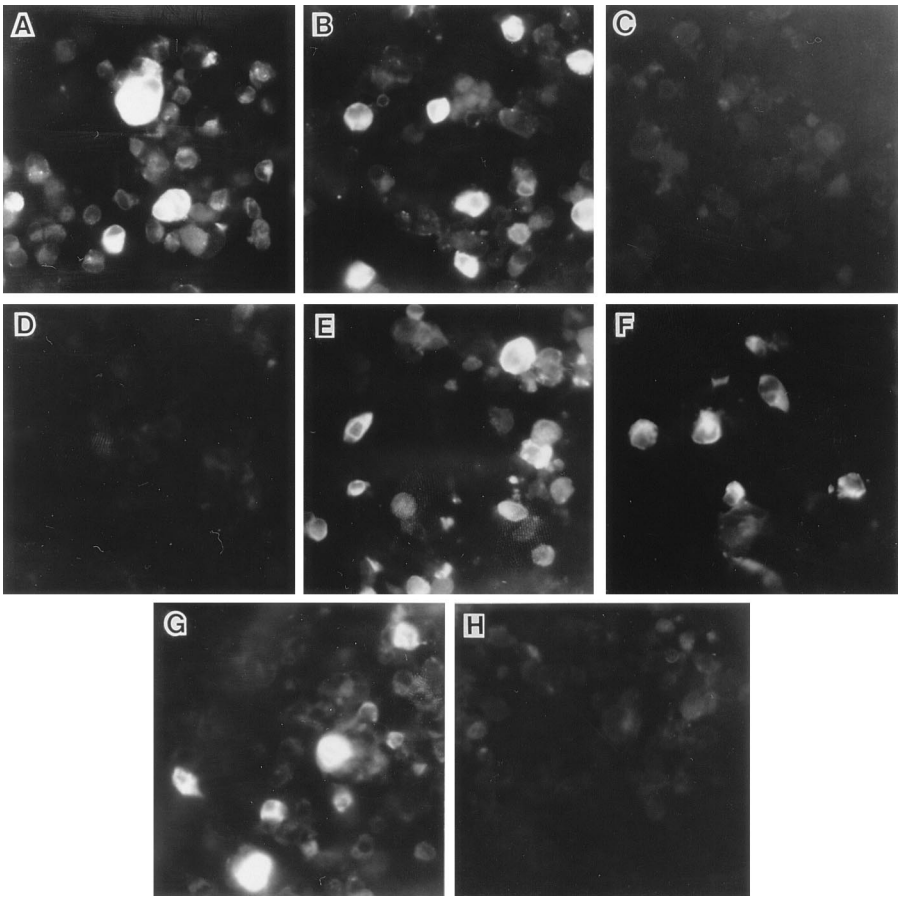

FIG. 3. Indirect immunofluorescence staining of CV-1 cells transfected with plasmid pTM1-gH (A and C), plasmid pTM1-gL (B and D), or plasmids pTM1-gH and pTM1-gL (E to H). Cells were fixed in ice-cold acetone and reacted with antipeptide antibody anti-gH (A and E), antipeptide antibody anti-gL (B and F), monoclonal antibody E1D1 (C, D, and G), or monoclonal antibody F-2-1 (H) and with anti-rabbit or anti-mouse antibody conjugated to fluorescein, as appropriate.

few or no methionine residues, a hydrophobic sequence that might function as a signal peptide, and three to four potential N-linked glycosylation sites. The best candidate was the BZLF2 ORF, which is predicted to encode a 223-amino-acid protein including no methionine residues, four N-linked glycosylation sites, a potential signal peptide but no other hydrophobic domain long enough to be membrane spanning. An antibody, anti-BZLF2, was made to a peptide corresponding to residues 71 to 88 of the BZLF2 ORF. Anti-BZLF2 immunoprecipitated glycoproteins from virus-producing cells which comigrated with the glycoproteins of 42,000 and 38,000 Da that were immunoprecipitated as part of the gH complex by antibody F-2-1 (Fig. 5). The BZLF2 ORF was then cloned for expression in the pTM1 vector (pTM1-BZLF2). Two proteins of the appropriate sizes that reacted in Western blots with anti-BZLF2 were made in CV-1 cells transfected with pTM1-BZLF2 and infected with recombinant vaccinia virus expressing the T7 polymerase, suggesting that both gp42 and gp38 were products of the BZLF2 gene (Fig. 6, lane 1). A smaller protein of approximately 28 kDa was also uniquely visible in cells transfected with pTM1-Z. This presumably represents some alternatively processed form of the protein and may be an artifact of the transfection system, as we have not observed a similar product in EBV-producing cells. Coexpression of pTM1-gH, pTM1-gL, and pTM1-BZLF2 then reconstituted the epitope recognized by antibody F-2-1 and facilitated immunoprecipitation of the entire gH-gL-gp42 complex from cells labeled with [^{35}S]cysteine (Fig. 6, lane 3). Curiously, however, if the proteins from transfected cells were separated by velocity sedimentation in sucrose to isolate any incompletely

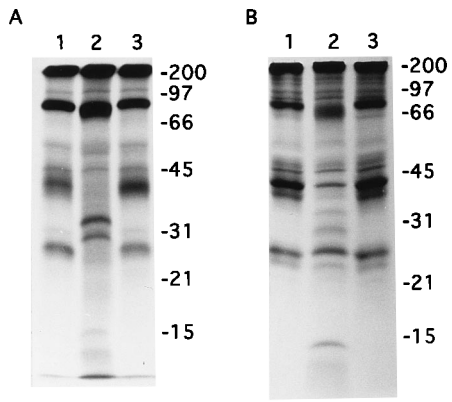

FIG. 4. SDS-PAGE analysis of proteins immunoprecipitated by antipeptide antibody anti-gL from Akata virus labeled with ^{125}I (A) or with induced Akata cells labeled with [^{35}S]cysteine (B) for 20 h at 6 h postinduction. Before electrophoresis, proteins were incubated overnight in digestion buffer alone (lanes 1), with endoglycosidase F (lanes 2), or with neuraminidase and *O*-glycanase (lanes 3). Sizes are indicated in kilodaltons.

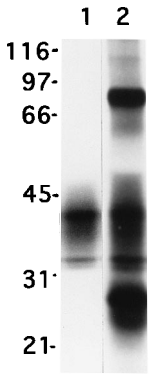

FIG. 5. SDS-PAGE analysis of proteins immunoprecipitated by antipeptide antibody anti-BZLF2 (lane 1) or monoclonal antibody F-2-1 (lane 2) from induced Akata cells labeled with [^{3}H]glucosamine for 20 h at 6 h postinduction. Sizes are indicated in kilodaltons.

complexed proteins (Fig. 6, lanes 5 to 9), antibody F-2-1 was revealed as being able to immunoprecipitate gp42 in the absence of its partners.

Reactivity of antibody F-2-1. To explore further the nature of the epitope recognized by antibody F-2-1, CV-1 cells were individually transfected with pTM1-gH, pTM1-gL, or pTM1-BZLF2 and reacted in indirect immunofluorescence assays with antipeptide antibodies anti-gH, anti-gL, and anti-BZLF2 or with monoclonal antibody F-2-1 (Fig. 7). The epitope recognized by F-2-1 was present on the BZLF2 gene product gp42. Reactivity of the antibody with the surface of transfected cells also indicated that gp42 was independently transported to the cell surface. Reactivity of native virus proteins in Akata cells was then reexamined by treating lysates of virus-producing cells with a sonic probe for 2 min prior to immunoprecipitation. Each of the three components of the complex could be immunoprecipitated in isolation by the appropriate antipeptide antibody or, in the case of gp42, by antibody F-2-1 (Fig. 8).

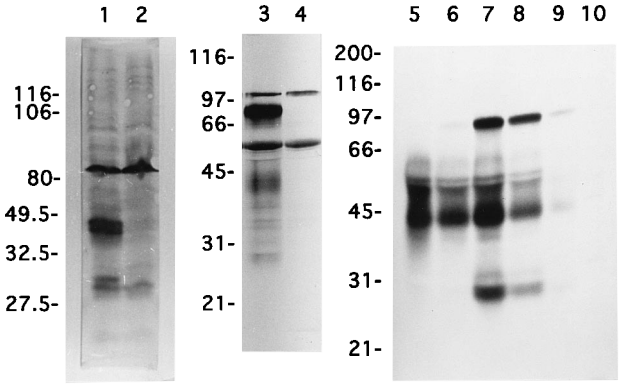

FIG. 6. Analysis of proteins expressed in cells transfected with plasmids pTM1 (lanes 2, 4, and 10), with plasmid pTM1-BZLF2 (lane 1), or with plasmids pTM1-gH, pTM1-gL, and pTM1-BZLF2 (lanes 3 and 5 to 9), infected with recombinant vaccinia virus expressing the T7 polymerase, and harvested at 22 h posttransfection without addition of label or after labeling for 18 h with [^{35}S]cysteine (lanes 3 and 4) or [^{3}H]glucosamine (lanes 5 to 10). Proteins in lanes 1 and 2 were analyzed by SDS-PAGE and Western blotting with antipeptide antibody anti-BZLF2. Proteins in lanes 3, 4, and 10 were immunoprecipitated with monoclonal antibody F-2-1 before SDS-PAGE analysis. Proteins in lanes 5 to 9 were sedimented through a gradient of 5 to 25% sucrose and separated into fractions before immunoprecipitation with antibody F-2-1. Fractions were numbered from the top of the gradient, and those shown are fractions 4 through 8 (lanes 5 to 9, respectively). Sizes are indicated in kilodaltons.

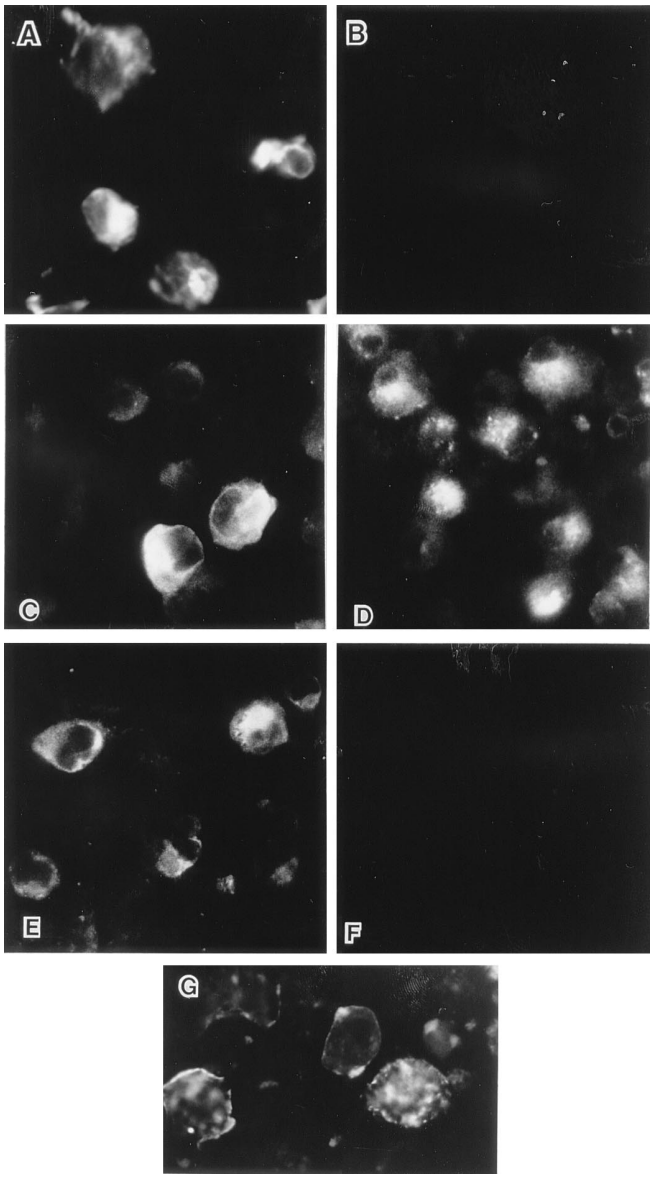

FIG. 7. Indirect immunofluorescence staining of CV-1 cells transfected with plasmid pTM1-gH (A and B), plasmid pTM1-BZLF2 (C, D, and G), or plasmid pTM1-gL (E and F). Cells were reacted with monoclonal antibody F-2-1 (B, D, F, and G) or with antipeptide antibody anti-gH (A), anti-BZLF2 (C), or anti-gL (E) and with anti-rabbit or anti-mouse antibody conjugated to fluorescein, as appropriate. Cells in panels A to F were fixed in ice-cold acetone before reaction with antibody. Cells in panel G were unfixed.

To explore further the interaction between different components of the gH complex, lysates of virus-producing cells were separated by velocity sedimentation through gradients of 5 to 25% sucrose, and each fraction was divided into two for immunoprecipitation. The antibodies used were F-2-1 and anti-gL, since anti-gH, the only antibody now available that can be certain to recognize an epitope on gp85-gH, immunoprecipitated gH alone but never gH associated with other members of the complex. Anti-gL immunoprecipitated gL alone in fraction 4 toward the top of the gradient, either because uncomplexed protein was present in the cells or because the complex was not completely stable under the conditions used (Fig. 9). The antibody immunoprecipitated a complex of gH and gL from the

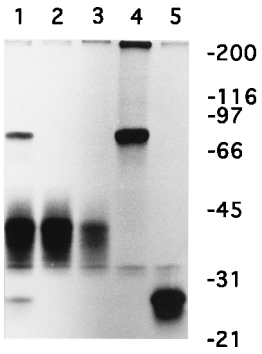

FIG. 8. SDS-PAGE analysis of proteins immunoprecipitated from induced Akata cells labeled for 20 h at 6 h postinduction with [^{3}H]glucosamine by antibody F-2-1 (lanes 1 and 2) or antipeptide antibody anti-BZLF2 (lane 3), anti-gH (lane 4), or anti-gL (lane 5). Proteins in lanes 2 to 5 were sonicated with a sonic probe for 2 min before addition of antibody. Sizes are indicated in kilodaltons.

TABLE 1. Effects of monoclonal antibodies F-2-1 and E1D1 on the ability of EBV to induce immunoglobulin synthesis in or transform T-depleted human peripheral leukocytes

Monoclonal antibody	Monoclonal antibody concn (μg/ml)	Immunoglobulin concn (ng/ml)				Transforming foci
		Expt 1	Expt 2	Expt 3	Expt 4	
F-2-1	1,000	162	0	0	0	–
	300	142	0	0	207	ND[a]
	100	604	0	560	541	ND
	30	2,075	40	1,215	1,409	ND
	10	4,920	1,500	809	3,256	ND
E1D1	1,000	6,200	6,025	1,464	3,040	+
	300	4,700	4,029	1,069	4,863	ND
	100	8,400	4,124	1,881	4,863	ND
	30	9,846	8,308	975	4,729	ND
	10	7,211	4,314	4,460	2,137	ND
None	0	8,100	3,269	1,146	4,457	+

[a] ND, not done.

faster-sedimenting fraction 5, immunoprecipitated gH, gp42/38, and gL from fractions 6 and 7, and immunoprecipitated gH, gp42, and gL from fraction 8. As expected, antibody F-2-1 did not immunoprecipitate gL alone, did not immunoprecipitate a complex consisting of gH and gL alone, and immunoprecipitated the gH-gp42/38-gL complex from fractions 6 through 8. Of greater interest, however, was the observation that antibody F-2-1 immunoprecipitated a complex of gp42/38 and gL from fraction 5 in the absence of gH. This finding suggested that the association of gp42/38 with the gH-gL complex involved primarily an interaction with gL and not gH. Electrophoresis of the complex in the absence of reducing agents did not alter the mobility of any of its components (data not shown), suggesting that interactions between them did not involve disulfide bonding.

Comparison of the effects of F-2-1 and E1D1 on infection of B cells and epithelial cells. The discovery that antibody F-2-1 reacted with the BZLF2 gene product gp42, and not with gH as had previously been assumed, coincided with an observation that the antibody had no effect on the ability of EBV to infect and induce EBNA production (specifically EBNA1 with an approximate M_r of 75,000) in the epithelial cell-derived SVK-CR2 cell line. A reexamination was then made of the effects of the two antibodies F-2-1 and E1D1 on infection of both B lymphocytes and SVK-CR2 cells. As previously reported, antibody F-2-1 neutralized the ability of EBV to induce immunoglobulin synthesis in freshly isolated T-cell-depleted leukocytes and inhibited the ability of virus to transform these cells, whereas E1D1 had no effect (Table 1). In contrast, although antibody 72A1, which reacts with the virus attachment protein gp350/220 and inhibits binding to the CR2 receptor, inhibited induction of EBNA1 in SVK-CR2 cells, antibody F-2-1 had no effect (Fig. 10). Further, antibody E1D1, despite its lack of effect on infection of B cells, completely inhibited EBNA1 induction in SVK-CR2 cells.

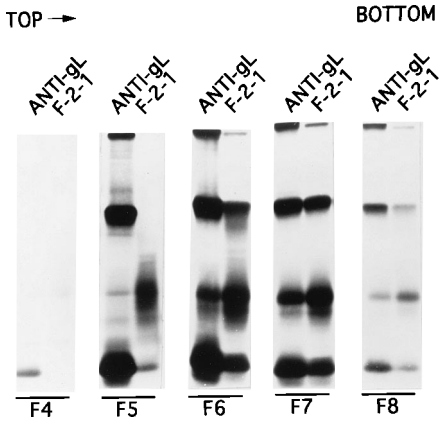

FIG. 9. SDS-PAGE analysis of proteins immunoprecipitated from induced Akata cells labeled with [^{3}H]glucosamine for 20 h at 6 h postinduction by antipeptide antibody anti-gL or monoclonal antibody F-2-1. Prior to immunoprecipitation, cell lysates were sedimented through a gradient of 5 to 25% sucrose and separated into 10 fractions. The fraction immunoprecipitated in each pair of lanes is indicated at the bottom (F4 [fourth from the top of the gradient] to F8 [toward the bottom of the gradient]).

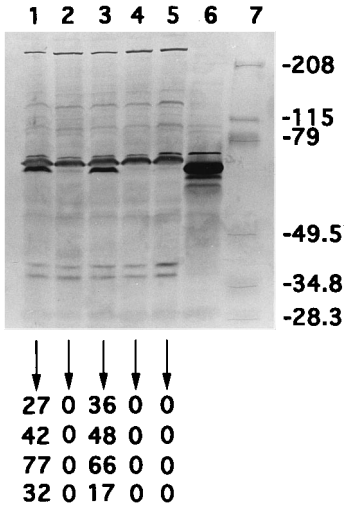

FIG. 10. SDS-PAGE and Western blot of SVK-CR2 cells (lanes 1 to 5) or uninduced Akata cells (lane 6). The blot was reacted with human serum containing antibodies to EBNA1. SVK-CR2 cells were infected with Akata virus preincubated with phosphate-buffered saline (lane 1) or with monoclonal antibody 72A1 (lane 2), F-2-1 (lane 3), or E1D1 (lane 4) or were left uninfected (lane 5). Lane 7 contains molecular weight markers (sizes are indicated in kilodaltons). The number below the arrow under each lane represents the percentage of EBNA-positive cells in each category of infected SVK-CR2 cells as judged by indirect immunofluorescence. The top row represents the percentage of EBNA-positive cells in the experiment used for the Western blot. The remaining rows represent results from three additional experiments.

DISCUSSION

Although in some respects EBV is a typical herpesvirus, it has evolved many unique strategies to infect and maintain a presence in lymphoid tissues of the body. It is the only herpesvirus that has been shown to attach to a target cell via a high-affinity protein-protein interaction (reviewed in reference 2), and results presented here indicate that EBV envelope proteins that are involved in penetration into B cells also include novel features. We have previously shown that gp85, the gH homolog of EBV, associates with gp25, which is the product of the BKRF2 ORF and the EBV homolog of gL (25). Coexpression of the two proteins in the vaccinia virus-pTM1 expression system, as described here, confirmed that they are capable of forming a stable complex in the absence of other virus glycoproteins. Separation of the native complex in sucrose gradients provided correlative data for the behavior of the authentic proteins in EBV-producing cells. Further, association of gH with gL resulted in transport of gH to the cell surface and restoration of the epitope recognized by antibody E1D1. The accessibility of gH to labeling with ^{125}I greatly increased in cells transfected with plasmids expressing both gH and gL. Although there was little change in labeling of gL under similar circumstances, the association of the protein with gH implied that it too moved to the cell surface in doubly transfected cells. The only antibody that unequivocally reacts with gL, the antipeptide antibody anti-gL, gave very high background staining against unfixed cells infected with vaccinia virus, and so it remains uncertain whether gL was transported to the cell surface in the absence of gH or whether labeling of the protein could be attributed to damage of the vaccinia virus-infected cell membrane. Since there was no reason to suspect greater loss of membrane integrity in cells transfected with plasmid expressing gL than with plasmid expressing gH, the more likely explanation would seem to be that the putative signal peptide is not cleaved and that it is not dependent on association with gH for transport to the cell surface. Apart from this possible difference, the behavior of the gH and gL homologs of EBV is in many respects similar to that of other herpesviruses (3, 9, 10, 12, 20, 21).

It was clear, however, that additional glycoproteins, a doublet of 42 and 38 kDa, could be coprecipitated with gH and gL and that the association was stable even in the presence of 0.1% SDS. Endoglycosidase digestion of the entire complex immunoprecipitated by antibody F-2-1 or anti-gL provided information that facilitated an educated guess as to the origin of the additional molecules. The ability of the antipeptide antibody anti-BZLF2 to immunoprecipitate glycoproteins that comigrated with the 42- and 38-kDa glycoproteins and the reactivity of the same antibody with both proteins in cells transfected with a plasmid expressing the BZLF2 ORF provided strong evidence that gp42 and gp38 were alternatively processed forms of the BZLF2 gene product. Restoration of the ability of antibody F-2-1 to immunoprecipitate gH and its associated proteins from cells cotransfected with pTM1-gH, pTM1-gL, and pTM1-BZLF2 provided further confirmation that the origins of the additional members of the EBV gH-gL complex had been correctly identified.

The finding that the epitope recognized by antibody F-2-1 is carried by the BZLF2 gene product was a surprise. The immunofluorescence data obtained with cells transfected individually with plasmids pTM1-gH, pTM1-gL, and pTM1-BZLF2 provided strong evidence for the possibility. Such reactivity would not, however, alone have been sufficient to make the assignment. Pulford and Morgan (14a) found that F-2-1 reacted in immunofluorescence assays with insect cells coexpressing gH and gL as recombinant baculovirus proteins, although they were unable to immunoprecipitate proteins with the same antibody. We could not detect any similar reactivity with gH and gL expressed in CV-1 cells, but rather in experiments in which the native gH complex was sonicated extensively before immunoprecipitation, we found convincing and reproducible evidence for the unique immunoprecipitation of gp42/38. The ability of the antibody to immunoprecipitate a complex of gp42 and gL, but not a complex of gH and gL, from lysates of virus-producing cells separated by velocity sedimentation in sucrose further supported the assignment of the epitope in authentic EBV protein to gp42/38 and not to an epitope defined by coexpression of gH and gL.

The reciprocal effect of antibodies E1D1 and F-2-1 on infection of lymphocytes and the epithelial line SVK-CR2 was also quite unexpected. Interpretation of this finding must be tempered by the fact that SVK-CR2 cells represent a somewhat artificial model of epithelial infection by EBV. However, although attachment of virus to these cells is mediated by the B-cell receptor for EBV expressed from a stably transfected cDNA clone, there is no compelling reason to believe that the penetration process differs from that which normally occurs in epithelial tissue. If this is indeed the case, it raises the intriguing possibility that the mechanisms of involvement of the gH complex in internalization of virus into the two cell types are different. We cannot legitimately extrapolate from the behavior of only two antibodies to an assignment of the roles of entire proteins. It is, however, tempting to speculate that just as EBV has evolved a protein, gp350/220, which is uniquely adapted for its attachment to B lymphocytes (16, 24), so may it have evolved a second protein, gp42/38, which is uniquely adapted for penetration into the same cell type. The apparent stability of the gH-gL complex in the absence of gp42 suggests that derivation of virus deleted for expression of the BZLF2 gene product might be a feasible approach to testing this hypothesis.

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