Characterization and Expression of a Glycoprotein Encoded by the Epstein-Barr Virus *Bam*HI I Fragment

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Computer-assisted analysis of the Epstein-Barr virus (EBV) open reading frame BILF2 (B95-8 nucleotides 150,525 to 149,782) predicts that it codes for a membrane-bound glycoprotein. [³H]glucosamine labeling of cells infected with vaccinia virus recombinants that expressed the BILF2 open reading frame revealed several diffuse species of glycoproteins of around 80,000 and 55,000 daltons. A monoclonal antibody derived from spleens of mice immunized with EBV immunoprecipitated the EBV-derived protein made by the vaccinia virus recombinants and also precipitated a late envelope glycoprotein with a mobility of 78,000 to 55,000 from EBV-producing cells. N-Glycanase treatment of the immunoprecipitated BILF2 product from EBV-producing cells resulted in a polypeptide of 28 kilodaltons, closely agreeing with the predicted molecular mass for the unmodified BILF2 gene product. Western (immuno-) blots using recombinant infected cells as a source of antigen showed that the majority of EBV-seropositive individuals have a serum antibody response to the BILF2-encoded gp78/55.

Epstein-Barr virus (EBV) is the causative agent of infectious mononucleosis (17), is thought to contribute to the development of lymphoproliferative syndromes in the immunosuppressed (8, 40), and is closely associated with two human malignancies, endemic Burkitt's lymphoma (9) and nasopharyngeal carcinoma (45). Although EBV may not be the sole or direct cause of these malignancies, it appears to be a significant risk factor in their development, and thus prevention of infection may be an effective means of controlling virus-associated neoplasms (10). The importance of attempts to develop an EBV vaccine is highlighted by the fact that an estimated 85,000 new cases of Burkitt's lymphoma and nasopharyngeal carcinoma arise annually (37).

Glycoproteins are of particular interest in studies aimed at producing virus vaccines because they are highly immunogenic and sera raised against them often neutralize virus. So far, three EBV glycoproteins have been characterized. These are specified by the open reading frames (ORFs) BLLF1, which encodes gp340/220 (4, 21), the virus attachment protein (34, 47); BALF4, which encodes gp110, the herpes simplex virus type 1 gB homolog (14, 38); and BXLF2, which encodes gp85, the herpes simplex virus type 1 gH homolog (16, 35). Much of the effort to produce a vaccine has focused on gp340/220 because monoclonal antibodies and polyclonal antisera against these proteins neutralize virus in vitro (20, 48, 49) and immunization with purified gp340 has protected cottontop tamarins against EBV-induced lymphomas (11). The efficacy and reliability of a vaccine is, however, likely to be improved if more than one protein is used as an immunogen. We have therefore attempted to identify other EBV glycoproteins with vaccine potential. Two approaches were taken. First, the EBV DNA sequence (2) and RNA mapping data (12) were analyzed for ORFs with potential for encoding integral membrane proteins with N-linked glycosylation sites, and candidate sequences expressed in vaccinia virus. Second, monoclonal

antibodies were selected for reactivity to virion envelope proteins and were used as tools to characterize the molecules that they recognized. We report here on a combination of the two approaches that enabled us to identify the product of the BILF2 ORF (nucleotides 150,525 to 149,782) as a late, virion-associated membrane glycoprotein with a mobility of 78,000 to 55,000 daltons. In accordance with the current practice of naming EBV glycoproteins, we refer to the molecule as glycoprotein gp78/55.

MATERIALS AND METHODS

Cells. Lymphoblastoid cell lines were grown in RPMI 1640 containing 10% heat-inactivated fetal calf serum. These cell lines included two human EBV genome-positive lines, the latently infected Raji cell line (39) and the EBV-producing cell line P3HR1-C113 (18); two EBV-producing marmoset cell lines, B95-8 (31) and MCV5 (a gift of George Miller, Yale University, New Haven, Conn.); and an EBV-negative human cell line, Ball 1 (19). Human thymidine kinase-negative 143B cells (41) were grown in minimal essential medium containing 25 µg of bromodeoxyuridine (BUDR) supplemented with 5% fetal calf serum per ml, and the monkey kidney cell line CV1 was grown in Dulbecco modified Eagle medium containing 10% fetal calf serum.

Virus. For growth of large stocks of vaccinia virus, HeLaS3 Spinner cells were maintained in Joklik suspension medium supplemented with 10% horse serum. The vaccinia virus strain WR and recombinants expressing the BILF2 ORF were grown and purified as described previously (22). EBV was harvested from the spent culture medium of producing cells 7 days after they had been induced with 30 ng of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) per ml. Media were centrifuged at 4,000 × *g* for 10 min to remove cells. Bacitracin (100 µg/ml) was added to the clarified supernatant, and the virus was pelleted by centrifugation at 20,000 × *g* for 90 min. Pellets were suspended in 1/250 the original volume of medium containing 100 µg of bacitracin per ml, reclarified by centrifugation three or four times at 400 × *g*,

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filtered (pore size, 0.8 μm ; Millipore Corp., Bedford, Mass.), and stored at -70°C . For some experiments, the virus was also further purified by centrifugation over dextran (33). The virus was repelleted and suspended in buffer containing 0.01 M Tris, 0.15 M NaCl, and 100 μg of bacitracin per ml at pH 7.2 (TNB). It was then layered over a discontinuous gradient of 5, 10, 15, and 30% Dextran T10 (Pharmacia, Inc., Piscataway, N.J.) in TNB and centrifuged at $76,000 \times g$ for 1 h. The virus was collected from the 15 to 30% interface, dialyzed against TNB to remove dextran, repelleted, and suspended in fresh TNB for use.

Hybridoma antibodies. Three hybridoma antibodies were used: 72A1 (20), which reacts with gp340/220; F-2-1 (46), which reacts with gp85; and E2A5, which was secreted by a hybridoma that was made from spleens of mice immunized with TPA-induced P3HR1-C1113 cells as previously described (46). All hybridoma antibodies were purified from culture supernatants by chromatography on protein A-agarose.

Antipeptide antibody. Antibodies to a synthetic peptide derived from residues 173 to 190 of the BILF2 sequence were made as previously described (35). Briefly, 400 μg of peptide was coupled via an N-terminal cysteine residue to keyhole limpet hemocyanin and emulsified in Freund complete adjuvant for subcutaneous injection into a rabbit. The rabbit was reinoculated four times at weekly intervals with the same amount of coupled protein emulsified in Freund incomplete adjuvant. Immunoglobulin G was purified from preimmune and immune sera by chromatography on protein A-agarose. Immune sera were further purified by affinity chromatography on peptide coupled to Affigel-10 beads (Bio-Rad Laboratories, Richmond, Calif.) according to the manufacturer's instructions. Bound immunoglobulin G was eluted from the Sepharose with 0.1 M acetic acid in 0.1 M NaCl. Reactivity of antisera with synthetic peptides was measured in enzyme-linked immunosorbent assays done in 96-well microdilution plates as previously described (35). Antibody to the BILF2 peptide reacted with plates coated with BILF2 peptide but not with plates coated with an irrelevant peptide. Preimmune sera did not react in either case.

Plasmid constructions. Routine procedures were carried out as described by Maniatis and colleagues (30). Plasmids were maintained in *Escherichia coli* DH5 α and purified by an alkaline extraction procedure (5). The EBV BILF2 ORF was excised as a 775-base-pair *DraI*-*RsaI* fragment from the *Bam*HI I clone of B95-8 virus DNA (1) and cloned into the *SmaI* site of pGS20 (28). Enzymes were purchased from various molecular biology companies and were used according to the instructions of the suppliers.

Generation of recombinants. Recombinants were obtained essentially as outlined previously (29). A plasmid vector was constructed which contains the vaccinia virus 7.5K promoter (28) upstream of the BILF2 ORF inserted into the body of the vaccinia virus TK gene. This plasmid (pBILF2) was then used to recombine the BILF2 gene into the vaccinia virus genome in a manner similar to that performed for marker rescue (51). CV1 cells were infected with wild-type, thymidine kinase-positive (TK⁺) vaccinia virus strain WR at a multiplicity of 0.1 to 0.5 PFU per cell. At 2 h postinfection, calcium phosphate-precipitated pBILF2 DNA was added, and cells were harvested at 48 h postinfection. Spontaneously generated TK⁻ virus and recombinant viruses were selected from infected-cell lysates by plaque assay on HTK⁻ 143B cells with 25 μg of BUdR per ml in the agar overlay. Total TK⁻ virus was then screened for BILF2

DNA by a dot blot hybridization protocol (27). Virus positive for BILF2 DNA was plaque purified twice and characterized more fully.

Radiolabeling and analysis of recombinant virus. Recombinant vaccinia viruses were grown in monolayers of HTK⁻ 143B cells (5×10^5 to 5×10^6) infected at a multiplicity of infection of 30 PFU per cell. After 2 h, the virus inoculum was replaced with minimal essential medium containing 50 μCi of [³H]glycosamine per ml. The cells were incubated for a further 6 h at 37°C followed by careful washing three times with ice-cold phosphate-buffered saline. Total cell extracts were made by adding 1 ml of $2\times$ disruption buffer (0.06 M Tris hydrochloride, pH 6.8, 3% sodium dodecyl sulfate [SDS], 5% β -mercaptoethanol, 10% glycerol, 0.02% bromophenol blue) directly to the monolayers. The extract was either stored frozen or a portion of it was diluted 1:1 with H₂O, and the proteins were fractionated by electrophoresis in a 7.5% SDS-polyacrylamide gel (24). After electrophoresis, the gel was fixed, treated with En³Hance (Dupont, NEN Research Products, Boston, Mass.), and fluorographed.

Radiolabeling for immunoprecipitation. EBV proteins were labeled extrinsically with ¹²⁵I (Amersham Corp., Arlington Heights, Ill.). Virus was pelleted from 9 ml of concentrated culture supernatant by centrifugation at $100,000 \times g$ for 1 h, suspended in 400 μl of phosphate-buffered saline, and labeled with 0.5 mCi of ¹²⁵I by use of Iodogen (tetrachlorodiphenylglycoluril; Pierce Chemical Co., Rockford, Ill.) (13). Labeled virus was then repelleted before solubilization. EBV-producing cells to be labeled metabolically with [³H]glucosamine (specific activity, 40 Ci/mmol; Amersham) were induced for 2 days with 30 ng of TPA per ml and then suspended in Hanks balanced salt solution supplemented with nonessential amino acids but with 1/10 the normal concentration of glucose, 2% dialyzed, heat-inactivated fetal calf serum, 30 ng of TPA per ml, and 100 μCi of [³H]glucosamine per ml. Cells were harvested 24 h after addition of label. In experiments which used phosphonoacetic acid, (Sigma Chemical Co., St. Louis, Mo.), cells were washed, suspended in medium containing 200 μg of phosphonoacetic acid per ml, incubated for a minimum of 2 days, washed again, and labeled with [³H]glucosamine as described above, but in the continued presence of phosphonoacetic acid. CV1 cells were infected with recombinant vaccinia viruses at a multiplicity of infection of 0.05 PFU per cell. Five hours after infection, medium was replaced with Hanks balanced salt solution supplemented with nonessential amino acids, but with 1/10 the normal concentration of glucose and 2% dialyzed, heat-inactivated fetal calf serum. One hour later, 100 μCi of [³H]glucosamine per ml was added, and cells were harvested after a further 18 h of incubation. Immunoprecipitations were carried out as previously described (3). Briefly, cells and virus were solubilized in radioimmunoprecipitation buffer containing 0.05 M Tris hydrochloride (pH 7.2), 0.15 M NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 0.1 mM phenylmethylsulfonylfluoride, and 100 U of aprotinin per ml, sonicated, and centrifuged at $100,000 \times g$ for 1 h. Supernatants were mixed with antibody and protein A-agarose beads (Genzyme Corp.). The precipitates were washed, dissociated by boiling in sample buffer containing 2-mercaptoethanol, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) in 9% acrylamide cross-linked with 0.28% *N,N'*-diallyltartardiamide. Molecular weight markers (Sigma) were electrophoresed in parallel tracks. Gels containing proteins labeled with ¹²⁵I were stained, destained, dried on filter paper, and placed in contact with XAR film (Eastman Kodak Co.,

Rochester, N.Y.) at -70°C for fluorography (6). Gels containing proteins labeled with [^3H]glucosamine were infused with 2,5-diphenyloxazole before being dried.

Enzyme digestion. Immunoprecipitated proteins bound to protein A-agarose beads were eluted by boiling the pellets for 3 min in 0.5% SDS–0.2 M sodium phosphate, pH 8.6–0.7% 2-mercaptoethanol and then were incubated at 37°C overnight in 0.55 M sodium phosphate, pH 8.6, containing 7.5% Nonidet P-40, 250 U of *N*-glycanase (Genzyme) per ml, and 100 nM 1,10-phenylanthroline hydrate in a final volume of 100 μl . Control samples without *N*-glycanase were incubated at either 37 or 4°C overnight. After incubation, 100 μl of sample buffer ($2\times$) was added to each, and the samples were boiled for 3 min before analysis by SDS-PAGE.

Western immunoblot analysis. After SDS-PAGE, proteins were transferred to nitrocellulose (7). Nonspecific binding of antibody to the filters was prevented by incubation for 1 h at 37°C (or overnight at 4°C) with 5% dried milk powder (BLOTTO) in Tris-buffered saline (TBS), pH 7.5. Filters or strips were then incubated with an appropriate dilution of the serum under test in BLOTTO either for 1 h at room temperature or overnight at 4°C . After three washes for 10 min each in TBS, the filters or strips were incubated with ^{125}I -labeled protein A or ^{125}I -labeled protein G diluted to 0.05 $\mu\text{Ci/ml}$ in BLOTTO-TBS for 1 h at room temperature and then washed three times in TBS, dried, and autoradiographed.

Immunofluorescence staining. For staining with rabbit antisera, slides bearing air-dried acetone- or formaldehyde-fixed B95-8 or Raji cells were incubated at 37°C for 1 h in a humidified atmosphere with serial dilutions of the sera under test. Slides were washed in phosphate-buffered saline three times for 10 min each and subsequently incubated for 1 h at 37°C in a humidified atmosphere with the appropriate dilution of fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (Sigma). For staining with hybridoma antibody E2A5, cells were briefly fixed in suspension with ice-cold 0.1% paraformaldehyde and reacted sequentially with mouse antibody and fluorescein-conjugated sheep anti-mouse immunoglobulin G (44). The ability of hybridoma antibody to react with virion envelopes was tested in an indirect immunofluorescence assay in which Raji cells with and without virus were incubated sequentially with mouse antibody and fluorescein-conjugated sheep anti-mouse immunoglobulin G.

EBV neutralization. EBV neutralization was carried out as described previously (32).

RESULTS

Reactivity of monoclonal antibody E2A5. A hybridoma antibody, E2A5, derived from spleens of mice immunized with EBV-producing cells, was found to react in indirect immunofluorescence assays with the surfaces of virus-producing cells of either human or marmoset origin but not with the surfaces of cells that were latently infected and did not produce virus. The numbers of cells in virus-producing cultures which were reactive with E2A5 antibody were similar to those reactive with antibodies 72A1 and F-2-1, which recognize gp340/220 and gp85. The fluorescence staining pattern was ringlike (Fig. 1A), with an intensity similar to that seen with antibodies to gp85 (not shown). Antibody E2A5 also reacted with virus bound to the surfaces of receptor-bearing Raji cells (Fig. 1B), indicating that it recognized a molecule that associated with the virus particle.

To identify the molecule recognized by E2A5, the anti-

FIG. 1. Indirect immunofluorescence staining of cells with antibody E2A5 and rabbit antibody to the BILF2 gene product. (A) TPA-induced P3HR1-Cl13 cells fixed with 0.1% paraformaldehyde and incubated sequentially with antibody E2A5 and fluorescein-conjugated sheep anti-mouse antibody. (B) Raji cells fixed with 0.1% paraformaldehyde and incubated sequentially with EBV, antibody E2A5, and fluorescein-conjugated sheep anti-mouse antibody. (C) TPA-induced B95-8 cells fixed with 0.1% paraformaldehyde and incubated sequentially with antiserum from rabbits vaccinated with BILF2 and fluorescein-conjugated goat anti-rabbit antibody.

body was used to immunoprecipitate proteins from purified ^{125}I -labeled EBV and from EBV-producing cells that had been labeled metabolically with [^3H]glucosamine. The antibody immunoprecipitated a protein from both sources which ran diffusely in 9% acrylamide cross-linked with *N,N'*-diallyltartardiamide with an apparent mobility of between 60 and 85 kilodaltons (kDa) (Fig. 2). The most heavily labeled region of the band approximated a molecule of 78 kDa (gp78). A second [^3H]glucosamine-labeled protein of approximately 55 kDa (gp55) was visible after long exposure of the gels. No difference was detected in the mobility of the protein immunoprecipitated from marmoset and human producer cells. Labeling of producer cells in the presence of phosphonoacetic acid completely eliminated reactivity (Fig. 2, lane 4), indicating that the molecule was a late viral protein. The facts that antibody E2A5 reacted with the surfaces of producing cells and with purified virus preparations or virus bound to receptor-positive cells and that it could be labeled extrinsically with ^{125}I suggested that gp78/55 was present in the virus envelope. To determine the amount and linkage of sugars on gp78/55, the molecule was immunoprecipitated from [^3H]glucosamine-labeled cells and digested with *N*-glycanase, which hydrolyzes the glycosylamine linkage of all types of asparagine-linked oligosaccharides. The mobility of the smallest band visible by SDS-PAGE analysis of digested material was approximately 28 kDa (Fig. 2, lane 7).

FIG. 2. SDS-PAGE analysis of protein immunoprecipitated by antibody E2A5 (lanes 2 through 7) or by protein A alone (lane 1) from EBV purified on a dextran gradient and labeled with ^{125}I (lanes 1 and 2) and from TPA-induced P3HR1-Cl13 cells labeled with $[^3\text{H}]$ glucosamine in the absence (lane 3) or presence (lane 4) of 200 μg of phosphonoacetic acid per ml. The N-glycanase susceptibility of $[^3\text{H}]$ glucosamine-labeled proteins immunoprecipitated from TPA-induced P3HR1-Cl13 cells was analyzed by incubating immunoprecipitates overnight at 37°C (lane 5) or 4°C (lane 6) or at 37°C in the presence of N-glycanase (lane 7). An arrow on the right indicates the position of the fastest-migrating band visible in lane 7. Molecular masses (in kilodaltons) are indicated to the right of the lanes.

This initial characterization of gp78/55 suggested that it might be the product of one of two ORFs in the EBV genome, namely the BDLF3 or the BILF2 ORF. The large amount of sugar found on the molecule favored the hypothesis that it was a product of the BILF2 ORF (Fig. 3), which includes a slightly larger number of potential N-linked glycosylation sites than does the BDLF3 sequence (2). A sequence representing residues 173 to 190 within this reading frame was then selected (36) for synthesis, and a rabbit antibody was made to it. The antibody was purified by affinity chromatography on immobilized peptide and tested for its ability to immunoprecipitate protein from ^{125}I -labeled virus. Immune antibody and not preimmune antibody from the same animal immunoprecipitated a protein that ran in approximately the same region of the gel as that immunoprecipitated by E2A5, although it had a much less diffuse distribution, perhaps because the sequence to which the antibody was made was only accessible in a subset of glycosylated molecules (Fig. 4).

Expression of the BILF2 sequence by vaccinia virus recombinants. The data obtained by the methods described so far were compatible with the hypothesis that gp78/55 was encoded by the BILF2 ORF. Confirmation of the hypothesis could, however, best be obtained by expression of the gene. A 775-base-pair *DraI*-*RsaI* fragment from EBV *Bam*HI-I containing the entire BILF2 coding sequence and 18 base pairs upstream of the initiating methionine (Fig. 3) was cloned into the *SmaI* site of pGS20. (RNA mapping data [12] indicates that the gene is not spliced.) The resulting plasmid pBILF2 contains the BILF2 coding sequence adjacent to the vaccinia virus 7.5K gene promoter cloned within the vaccinia virus TK gene. CV1 cells infected with vaccinia virus

FIG. 3. Computer-assisted analysis of the EBV ORF BILF2 is shown together with the predicted amino acid sequence of the protein encoded by this ORF. The *DraI* and *RsaI* sites used in the cloning (see text) are indicated. Potential N-linked glycosylation sites are shown (*) below the amino acid sequence. Solid underlines indicate hydrophobic residues likely to be membrane-spanning regions. Nucleotides numbered 1 to 780 correspond to nucleotides 150,547 to 149,767 of B95-8 virus DNA (2).

were transfected with the plasmid construct, cells were harvested, and TK⁻ virus was isolated by plaque assay on TK⁻ cells in the presence of 25 μg of BUdR per ml. Recombinant viruses, TK⁻ by virtue of insertion of the BILF2 gene, were identified by screening for the presence of BILF2 gene DNA. A recombinant virus positive for BILF2 DNA was designated vBILF2 and plaque purified once more in TK⁻ cells in the presence of BUdR, and then large stocks were prepared under nonselective conditions in HeLa S3 spinner cells.

The DNA sequence of the BILF2 gene predicts a 248-residue polypeptide of approximately 27 kDa (Fig. 3). However, as BILF2 has the potential for 12 N-linked glycosylation sites, we expected a larger glycoprotein. An initial characterization of the BILF2 gene product obtained by using $[^3\text{H}]$ glucosamine to label the recombinant virus-infected cells is shown in Fig. 5A. The BILF2 gene product appears as a diffuse band of approximately 80 kDa. A Western blot of vBILF2-infected cell extracts probed with a pool of sera from individuals who were seronegative for vaccinia virus and seropositive for EBV is shown in Fig. 5B. A more diffuse band of approximately 75,000 to 80,000

FIG. 4. SDS-PAGE analysis in 9% acrylamide of proteins immunoprecipitated from ^{125}I -labeled EBV by antibody E2A5 (lane 1), preimmune rabbit antibody (lane 2), and antibody made after immunization of the rabbit with a peptide derived from the BILF2 sequence and affinity purified on peptide coupled to Affigel (lane 3).

daltons was observed, indicating that an antibody response to BILF2 is induced upon natural infection with EBV. When SDS-PAGE with gels containing a higher percentage of polyacrylamide was used to analyze the products, a second species of glycoprotein unique to vBILF2 recombinant-infected cells was observed migrating between 50,000 and 61,000 daltons (data not shown).

Immunoprecipitation of the recombinant virus proteins with antibody E2A5. To establish the identity of the BILF2 gene product and the glycoprotein immunoprecipitated from EBV by E2A5, the antibody was used to immunoprecipitate protein from CV1 cells infected with vBILF2. Cells infected with a recombinant virus expressing the envelope protein of the Friend leukemia virus (a gift of Edward Stephens, University of Florida) were used as a control. A glycosylated protein with a mobility similar to that of native gp78/55 was precipitated from the vBILF2-infected cells and not from cells infected with the unrelated recombinant (Fig. 6), confirming that gp78/55 is a product of the BILF2 gene.

Immune responses to the BILF2 protein induced by the recombinant BILF2 protein. Monoclonal antibody E2A5 had no effect on the infectivity of virus. To determine whether the vBILF2 recombinant could elicit neutralizing antibodies and provide additional reagents for examining the function of the authentic BILF2 protein, two rabbits were inoculated intradermally with 10^8 PFU of vBILF2. After vaccination, the rabbits developed typical local skin lesions, and at 28 days they were revaccinated intradermally with 10^8 PFU of vBILF2. No lesions were observed after this second vaccination. Serum from these rabbits gave a pattern of reactivity

FIG. 5. Analysis of the BILF2 ORF gene product expressed by a vaccinia virus recombinant. (A) HTK⁻ 143B cells were infected at a multiplicity of infection of 30 PFU per cell with two independent isolates of a recombinant vaccinia virus expressing the EBV ORF BILF2 (lanes 1 and 2) or vMA5, a recombinant expressing the EBV glycoprotein gp340 (lane 4). At 2 h postinfection or 2 h after mock infection (lane 3), the cells were labeled with [^3H]glucosamine. Total cell extracts were fractionated by electrophoresis on a 6% SDS-polyacrylamide gel. The gel was fixed and fluorographed. Molecular weight markers were run in parallel, and their positions are indicated on the right. HA indicates the migration of vaccinia virus hemagglutinin. (B) HTK⁻ 143B cells were mock infected (lane 3) or infected at 30 PFU per cell with recombinant vaccinia viruses expressing either the BILF2 gene product (lanes 1 and 2) or EBV gp340 (lane 4). At 18 h postinfection or 18 h after mock infection (lane 2), total cell extracts were fractionated on 7.5% SDS-polyacrylamide gels and transferred to nitrocellulose. Molecular weight markers were run in parallel, and their positions are indicated. The blots were incubated with a 1:50 dilution of a pool of sera from individuals who were negative for vaccinia virus antibody and EBV seropositive. Binding of antibody was detected by ^{125}I -labeled protein G and autoradiography.

with EBV-producing cell lines identical to that shown by the monoclonal antibody E2A5 (Fig. 1C), further confirming a surface location for gp78/55, characteristic of membrane-anchored glycoproteins. Although sera from animals vaccinated with a vaccinia virus gp340 recombinant neutralized EBV infectivity (26), sera from the vBILF2-vaccinated animals failed to neutralize EBV as assayed by immortalization of fetal cord lymphocytes (data not shown).

The BILF2-encoded protein does not appear to be abundant in EBV-producing cells. Cells infected with vBILF2 were therefore used as a source of BILF2 antigen in Western blots to analyze the responses of individuals to the protein after natural infection. Sera were obtained from 20 individuals who were born after routine vaccination with vaccinia virus was stopped. The reactivities of sera from a typical subset of these individuals is shown in Fig. 7. A few of these individuals reacted very strongly (e.g., Fig. 7, lane 3), and positive reactions could be detected at serum dilutions of greater than 1 in 500. Others reacted more weakly, while some were negative (e.g., Fig. 7, lane 6; this serum was also negative by immunofluorescence for anti-EBV virus capsid antigen antibody). In general, the anti-BILF2 antibody titers

FIG. 6. SDS-PAGE analysis in 9% acrylamide of [^3H]glucosamine-labeled proteins immunoprecipitated by antibody E2A5 from CV1 cells infected with vBILF2 (lane 1), from CV1 cells infected with a vaccinia virus recombinant expressing the envelope gene of the Friend leukemia virus (lane 2), or from TPA-induced P3HR1-Cl13 cells (lane 3). This figure is a composite made from two photographs of the same gel exposed for either 11 days (lanes 1 and 2) or 5 weeks (lane 3). Molecular weight markers are shown on the right.

as judged by reactivity on Western blots were paralleled by anti-VCA antibody titers as determined by immunofluorescence. Thus, the majority of, and possibly all, EBV-seropositive individuals produce antibody to BILF2.

DISCUSSION

RNA mapping data (12) and computer-assisted analysis (2, 12) (Fig. 5) suggested that a late viral glycoprotein produced from an unspliced message with the characteristics of an integral membrane protein is encoded by the EBV ORF BILF2. We have used a combination of approaches to demonstrate that this ORF is translated and codes for a molecule that is present in the virus envelope and in the membrane of EBV-producing cells.

A monoclonal antibody, E2A5, raised against the native molecule immunoprecipitated several species of glycoprotein both from [^3H]glucosamine-labeled lymphoblastoid cells which were producing EB virus and from cells which had been infected with a vaccinia virus recombinant. *N*-Glycanase treatment of material immunoprecipitated by E2A5 indicated that the mature BILF2 glycoprotein is produced from a 28-kDa precursor and contains little O-linked glycosylation. The major species of glycoprotein made by EBV-producing cells ran in SDS-polyacrylamide gels as a diffuse band with a molecular weight between 60,000 and 85,000; the predominantly labeled species was of approximately 78 kDa. A minor, quite distinct species of approximately 55 kDa was also observed when gels were exposed for several weeks. In contrast, although in some cell lines the vaccinia virus

FIG. 7. Western blot analysis of the immune response to the BILF2 gene product after natural infection with EBV CV1 cells infected with 20 PFU of a recombinant expressing the BILF2 ORF per cell for 18 h. Total cell extracts were run on 12.5% SDS-polyacrylamide gels and transferred to nitrocellulose. Strips cut from these blots were then incubated with 1:50 dilutions of sera from individuals (lanes 1 through 9) who had not come into contact with vaccinia virus. The individual represented in lane 6 was also EBV seronegative. Binding of antibody to strips was detected by using [^{125}I]labeled protein A and overnight autoradiography. In order to enhance the signal, lanes 6 through 9 have been exposed to film for 72 h.

BILF2 recombinant also produced more of the 78-kDa species than it did of the 55-kDa species, in others approximately equal amounts of the two were found. As vaccinia virus does not splice mRNA, these related proteins probably arise by posttranslational modification and not from different mRNA species. Differences in the relative proportions of the glycoproteins are likely to be due to the capacities of different cell lines to carry out posttranslational modifications such as glycosylation and protease cleavage.

Earlier characterizations of EBV envelope glycoproteins made with polyclonal sera provided no clues to the existence of gp78/55. This probably reflects the facts that the molecule runs diffusely in polyacrylamide gels, that it is present in low abundance in the virus, and that it is difficult to label metabolically. To date, we have been unable to visualize the protein by labeling with any of the nonessential amino acids. Labeling with [^{35}S]methionine is understandably poor, since the BILF2 sequence contains only one methionine residue. We have also found it impossible to label the native protein detectably with valine, threonine, or lysine.

There is no indication as yet of what function, if any, gp78/55 might play in the infectious cycle of the virus. Neither monoclonal antibody E2A5 nor polyclonal sera raised to the recombinant molecule neutralized the ability of EBV to transform cells. This might reflect the quality or titer of the sera. Although the 7.5K promoter used in the recombinant vaccinia virus is active at both early and late times in infection, the fact that BILF2 contains a signal (TTTTNT) (43) for termination of early transcription may mean that presentation of the BILF2 antigen is impaired. In recombinants expressing influenza hemagglutinin from a late promoter, the hemagglutinin was incorrectly presented to the immune system of infected mice, probably because of production of vaccinia virus-derived serine protease inhibitors (23). However, it is also possible that p78/55 plays no role in infection. Preliminary results obtained with virocytes made from EBV envelope proteins indicated that although deple-

tion of glycoprotein gp85 abrogated the ability of bound viroosomes to fuse with cells (15), depletion of gp78/55 had no effect on either binding or fusion (data not shown). Other herpesviruses are known to incorporate glycoproteins that are nonessential for growth in tissue culture into the virion envelope (25, 42, 50). Thus, gp78/55 may be a similarly redundant molecule. It is also possible that the glycoprotein plays a role in the egress of virus, which would not be detected by a transformation assay, or that it is of importance to replication of virus in epithelial rather than lymphoblastoid cells.

Whether or not gp78/55 plays a crucial role in virus replication, it is clearly synthesized *in vivo*, as a majority of seropositive individuals appear to make antibody to it. It remains to be seen whether it has diagnostic significance and whether or not its incorporation into a polyvalent vaccine might promote clearance of virus by antibody-dependent mechanisms.

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