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Identification of a Novel Protein Encoded by the *Bam*HI A Region of the Epstein-Barr Virus

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An unusual set of 3′ coterminal, spliced mRNAs transcribed through the *Bam*HI A fragment have been previously identified in nasopharyngeal carcinoma (NPC) tissues. These RNAs have also been detected at low levels in Burkitt's lymphoma (BL) cell lines and Epstein-Barr virus (EBV)-transformed lymphocytes. Sequence analyses of clones from a cDNA library derived from the C15 NPC tumor indicated that the primary transcripts are differentially spliced, giving rise to a family of related transcripts, all of which encompass the BARF0 open reading frame (ORF) at the 3′ end of the transcripts. One cDNA was identified that extended the BARF0 ORF at the 5′ end, forming the RK-BARF0 ORF. In this study, a rabbit antiserum to a synthetic peptide representing an amino acid sequence encoded by the BARF0 ORF was prepared. This antiserum detected a glutathione *S*-transferase–BARF0 fusion protein and both BARF0 and RK-BARF0 proteins expressed from transfected constructs in H1299 cells. The serum also immunoprecipitated the 20-kDa BARF0 and 30-kDa RK-BARF0 in vitro-translated proteins. Immunoblot analyses identified a protein doublet of 30 and 35 kDa in all of the EBV-infected cell lines tested. Cellular fractionation studies revealed that the proteins were membrane associated. The sizes of the proteins detected in cell lines and their association with membranes suggests that they are likely encoded by the RK-BARF0 transcript, which is predicted to contain a membrane localization signal. The proteins were also detected in protein extracts prepared from NPC biopsies and a BL biopsy but not from hairy leukoplakia, a permissive EBV infection. These results reveal that the rightward RNA transcripts from the *Bam*HI A region of EBV encode one or more proteins that are expressed in latently infected cells and in tumor tissue.

Epstein-Barr virus (EBV) is a herpesvirus that infects more than 90% of the human population worldwide. EBV can infect B lymphocytes and causes the B-cell lymphoproliferative diseases, acute infectious mononucleosis, and posttransplant lymphoma (20, 23, 34). EBV is also linked to endemic Burkitt's lymphoma (BL) (13, 48). Infection of B lymphocytes with EBV in vitro results in immortalized lymphoblastoid cell lines (LCLs) (30, 31). These infections are usually nonpermissive, with expression of only a limited number of viral genes. The proteins expressed in this type of latent infection include a family of EBV nuclear antigens, EBNA1, -2, -3A, -3B, -3C, EBNA leader protein (EBNA-LP), and three membrane proteins (LMP1, LMP 2A, and LMP2B). Three types of latency have been described for lymphoid cell lines and infected tissues (37). The pattern of expression detected in latently infected lymphocytes is termed latency III. In some BL cell lines, viral expression is tightly restricted and only the EBNA1 protein is detected. This level of expression has been called latency I.

EBV also can infect epithelial cells and is detected in the malignant epithelial cells in nasopharyngeal carcinoma (NPC), a tumor that occurs with high incidence among southern Chinese and Eskimos (9, 10, 33, 48). It has been shown that EBV-associated NPC represents a clonal expansion of a single EBV-infected progenitor cell, a finding that implies an etiologic role for the virus in these carcinomas (32). The EBV genes encoding EBNA1, LMP1, and LMP2 are consistently transcribed in NPC, a pattern of expression that is designated latency II (6, 11, 45). In addition, a family of rightward transcripts from the *Bam*HI A region were originally identified in NPC, where they are abundantly expressed (4, 7, 16, 17, 21). Sequence analyses of clones obtained from a cDNA library of tumor xenograft C15 revealed that the transcripts are differentially spliced, giving rise to a family of related transcripts in NPC tissues that are 3′ coterminal (38, 40). All of the *Bam*HI A transcripts contain the open reading frame (ORF) *Bam*HI A rightward frame 0 (BARF0) at their 3′ ends, which is predicted to encode a 174-amino-acid (aa) protein. One cDNA that extended this ORF at the 5′ end, forming the RK-BARF0 ORF, which would encode a 279-aa protein, was obtained (38).

Previous studies using PCR analysis and Northern blot hybridization detected *Bam*HI A transcripts in EBV-positive BL cell lines as well as in all EBV-transformed LCLs studied (7, 22, 38). Expression in established LCLs occurred irrespective of their virus-producing status and was not a consequence of continued in vitro passage, as spliced *Bam*HI A transcripts could be amplified from normal B cells within 1 day of experimental infection (4). However, the steady-state level of transcription in lymphoid cells was significantly lower than that in NPC (7, 22, 38, 40). It has been postulated that the *Bam*HI A transcripts, to maintain the latent state of cells, may act as antisense RNAs interfering with the expression of *Bam*HI A lytic cycle genes present on the complementary DNA strand (22). The alternately spliced *Bam*HI A RNAs contain several potential ORFs that may encode one or more novel proteins,

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and in vitro-translated BARF0 protein was recognized by NPC serum, suggesting that the *Bam*HI A transcripts encode a protein that is expressed in vivo (17).

In this study a peptide corresponding to a region of the putative BARF0 protein was synthesized and used to produce a BARF0-specific antiserum. This anti-BARF0 antiserum reacted with 30- and 35-kDa proteins in EBV-positive cell lines and in EBV-positive tumor biopsies. These data indicate that one of the rightward RNA transcripts from the *Bam*HI A region of EBV encodes a protein that is expressed in latent infection and in EBV-infected tumors in vivo.

MATERIALS AND METHODS

Cell lines. CB5 and SFC4 are human LCLs established by infection of cord blood lymphocytes with either B95-8 virus or virus from throat washings from human immunodeficiency virus (HIV)-positive patients, respectively. Mutu I and Mutu III are two different clones of an EBV-positive BL cell line representing type I latency and type III latency, respectively (19). Raji (25) and Akata (41, 42) are EBV-positive BL cell lines exhibiting type III latency and type I latency, respectively. Ramos is an EBV-negative BL cell line, BJAB is an EBV-negative high-grade B-cell lymphoma, and Jurkat is an EBV-negative T-cell lymphoma line. Lymphoid cell lines were maintained in RPMI 1640 medium containing penicillin and streptomycin and supplemented with 10% fetal bovine serum. The C33A cell line was derived from a human cervical carcinoma, and the H1299 cell line was derived from a nonsmall cell lung carcinoma. Both cell lines were maintained in Dulbecco's modified Eagle's medium containing penicillin and streptomycin and supplemented with 10% fetal bovine serum.

Clinical samples. C15 is an NPC tumor xenograft that has been serially passaged in athymic nude mice (5, 6). Four out of the five primary NPC biopsies were obtained from Chinese patients living in Malaysia, and one biopsy was obtained from an American Caucasian patient at University of North Carolina Hospitals. All NPC tumors were identified clinically, were confirmed by histopathology, and were classified as type I, II, or III according to the World Health Organization (WHO) guidelines. Upon collection, the biopsy samples were immediately placed in liquid nitrogen. The EBV-positive BL biopsy was clinically diagnosed, and its EBV status was determined by Southern blot analysis. Both squamous cell carcinomas (SCCs) were identified clinically, were confirmed by histopathology, and were found to be EBV negative by Southern blot analysis. Tongue biopsies were obtained from three HIV-positive patients with clinically and pathologically identified oral hairy leukoplakia (OHL) as well as from three clinically normal individuals. The presence of EBV in the OHL biopsies was confirmed by Southern blot analyses.

Antibody production. A peptide (CTDSMAARVPIEELRELR) from the amino-terminal portion of the putative BARF0 protein was synthesized and conjugated to keyhole limpet hemocyanin (KLH). Rabbits were immunized with this peptide conjugate by Immuno-Dynamics Inc. (La Jolla, Calif.). Antisera were screened for reactivity to a glutathione *S*-transferase (GST)-BARF0 fusion protein by immunoblotting.

Immunoblotting. Cells (2×10^7) were collected by centrifugation, resuspended in 400 μl of sodium dodecyl sulfate (SDS) sample buffer (5% [wt/vol] sodium lauryl sulfate, 25 mM Tris hydroxymethyl aminomethane [pH 6.8], 5% [vol/vol] 2-mercaptoethanol), and lysed by sonication. Cell samples were then heated to 100°C for 5 min, followed by centrifugation at 15,000 × *g* for 5 min. Supernatants were collected, and 20-μl samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Tumor and OHL biopsies were dispersed in 400 μl of sample buffer by sonication, and the mixtures were heated to 100°C for 5 min and then were centrifuged at 15,000 × *g* for 5 min. Supernatants were collected, and 20-μl samples were subjected to SDS-PAGE. Equal protein loading was confirmed by sample analysis SDS-PAGE and staining of the gel with Coomassie brilliant blue (Sigma). Following SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose filters (Schleicher and Schuell) with a semidry transfer apparatus (Hoefer). Filters were blocked by incubation in Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat dried milk (BLOTTO). Antigens were detected with either anti-EBNA1 (1:1,000) (8), anti-LMP1 S12 (1:10), or anti-FLAG M2 (1:350) monoclonal antibodies (Eastman Kodak Co.) or anti-BARF0–anti-RK-BARF0 rabbit sera (1:2,000) followed by incubation with horseradish peroxidase-conjugated antirabbit or antimouse antibody (Amersham) and were visualized by enhanced chemiluminescence according to the manufacturer's specifications (Amersham).

Production of the GST-BARF0 fusion protein. The BARF0 gene was obtained by PCR amplification of the BARF0 ORF from C15 DNA. Amplification was performed using primers containing *Bam*HI (5′-ATCACGAGGGATCCATGG CCGCCACCCTCCCCCTG-3′) and *Bgl*II (5′-ATCACGAGAGATCTTTAAT GAAGAGAATCAATAGC-3′) restriction enzyme sites to facilitate cloning. PCR was performed for 35 cycles, with annealing at 55°C for 30 s, extension at 72°C for 30 s, and denaturation at 94°C for 60 s. The amplified BARF0 was ligated into the multiple cloning site of the pGEX-2TK vector (Pharmacia). DNA sequencing confirmed the integrity of the BARF0 sequence. pGEX-2TK/BARF0 and pGEX-2TK were used to transform *Escherichia coli* DH5α cells, which were induced with isopropyl-β-D-thiogalactopyranoside (Sigma). The GST-BARF0 fusion protein was purified by absorption to glutathione-Sepharose agar (Pharmacia). The agarose beads were washed, resuspended in SDS sample buffer, boiled, and centrifuged, and the supernatant was subjected to SDS-PAGE.

Transient expression of BARF0-FLAG. The FLAG epitope was inserted into the amino termini of both the BARF0 and RK-BARF0 ORF products by using PCR primers to align the BARF0 and RK-BARF0 ATGs in frame with the FLAG ATG. The entire RK-BARF0 ORF was constructed by PCR amplification of two partially overlapping cDNAs, the 1.4-kb C25a cDNA (17) and the RK1 cDNA (38), that were cut with *Acc*I, ligated together, and used as a template for additional PCR amplification with the 3′ oligonucleotide primer incorporating the BARF0 translation stop codon. The BARF0 and RK-BARF0 PCR products were cut with *Bam*HI and *Bgl*II and were ligated into the pSG5-FLAG expression vector. The pSG5-BARF0-FLAG and pSG5-RK-BARF0-FLAG expression vectors, containing the FLAG-BARF0 or FLAG-RK-BARF0 sequence under control of the simian virus 40 early promoter, were transfected into the H1299 cell line using lipofectin reagent (Gibco BRL). At 48 h after transfection confluent cells were scraped from tissue culture dishes into SDS sample buffer. Cell extracts were subjected to SDS-PAGE and were immunoblotted with either anti-BARF0 or anti-FLAG antisera.

In vitro translation of BARF0 and RK-BARF0 proteins. The BARF0 and RK-BARF0 cDNAs were cloned into the pSG5 vector which contains a T7 promoter. [^{14}C]leucine-radiolabelled BARF0 and RK-BARF0 proteins were synthesized in vitro with the TNT T7 reticulocyte lysate system (Promega, Madison, Wis.). The radiolabelled in vitro-translated proteins were immunoprecipitated with the anti-BARF0 rabbit serum or control rabbit serum for 2 h at 4°C. The immune complex was collected by incubation with pansorbin for 16 h at 4°C followed by centrifugation at 15,000 × *g* for 5 min. The immunoprecipitate was then washed three times with Tris-buffered saline containing 0.1% Tween 20 (TBST) buffer, followed by two washes in 2× TBST and finally one wash in TBST containing 0.1% SDS. The precipitates were solubilized in SDS buffer, heated to 100°C for 5 min, and subjected to SDS-PAGE, and radiolabelled protein was visualized by fluorography.

Cell fractionation. CB5 cells were pelleted, washed with phosphate-buffered saline, and resuspended in HEM buffer (20 mM HEPES, 1 mM EDTA, 1 mM β-mercaptoethanol, and the protease inhibitors aprotinin, leupeptin, and phenylmethylsulfonyl fluoride). The cells were lysed by Dounce homogenization, and nuclei were collected by centrifugation at 750 × *g* for 5 min. Cell extracts were kept at 4°C for 5 min, and any remaining intact nuclei were again collected by centrifugation at 750 × *g* for 5 min. The supernatant was collected, and a crude membrane fraction was obtained by centrifugation at 43,000 × *g* for 20 min. The remaining supernatant represented the cytoplasmic fraction. The nuclear and membrane fractions were dispersed directly into SDS sample buffer, whereas protein in the soluble fraction was first precipitated by the addition of six volumes of acetone before being solubilized in SDS sample buffer. Equal protein loading on SDS-PAGE gels was determined by staining gels with Coomassie brilliant blue.

RESULTS

Generation of BARF0-specific antisera. On the basis of the predicted amino acid sequence encoded by the BARF0 ORF, an 18-mer peptide was synthesized. To allow convenient coupling of the peptide to KLH, the peptide was produced beginning with an N-terminal cysteine. The peptide-KLH conjugate was used to raise antisera in rabbits, and serum samples were assayed for reactivity to a GST-BARF0 fusion protein by immunoblotting. Serum from one rabbit (2161) reacted with the GST-BARF0 fusion protein but not with GST alone (Fig. 1A).

A FLAG epitope was added to the amino termini of both the BARF0 and RK-BARF0 ORF products by PCR. These constructs were used to confirm the specificities of the antisera by transient expression of the FLAG epitope-tagged constructs in H1299 cells. The BARF0-FLAG fusion protein was detected as a 22-kDa protein by immunoblotting with both the anti-FLAG antiserum and rabbit 2161 serum (Fig. 1B). Immunoblotting with both the anti-FLAG antiserum and rabbit 2161 serum also detected the RK-BARF0-FLAG fusion protein (Fig. 1C). These data indicate that rabbit 2161 serum reacts with the BARF0 and RK-BARF0 proteins expressed in vivo and reveal an absence of reactivity in cells transfected with the control vector.

The BARF0 and RK-BARF0 ORFs were translated in vitro in the presence of [^{14}C]leucine, and the products were immu-

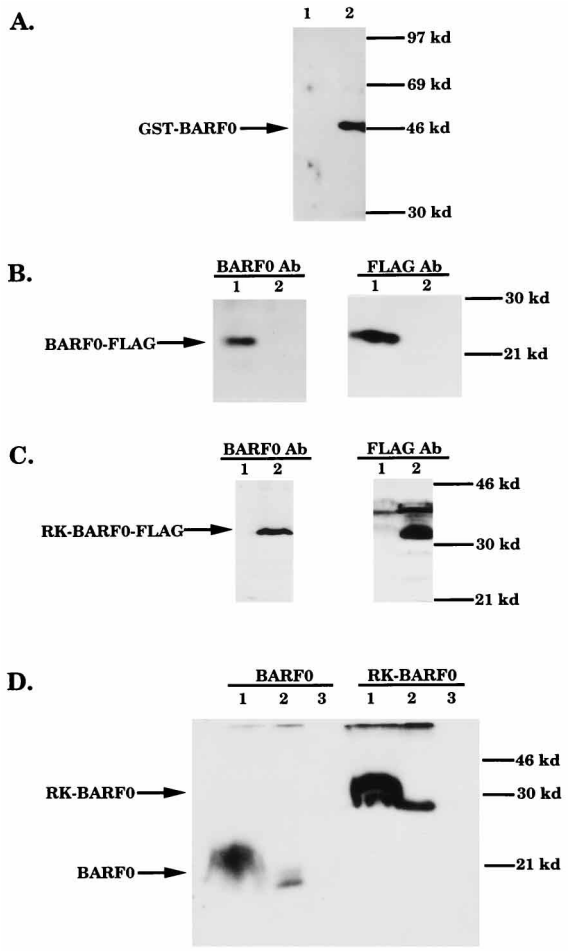

FIG. 1. Reactivity of rabbit serum to BARF0 and RK-BARF0 proteins. Serum from rabbit 2161 was used to detect a GST-BARF0 fusion protein (A) and transiently transfected BARF0 (B), transiently transfected RK-BARF0 (C), and in vitro-translated BARF0 and RK-BARF0 proteins (D). In panel A GST (lane 1) and GST-BARF0 (lane 2) were subjected to SDS–10% PAGE, immunoblotted, and reacted with rabbit 2161 serum. In panel B the BARF0 gene, which contains additional nucleotides encoding the FLAG sequence, was transiently transfected into H1299 cells (lane 1) as well as the vector control (lane 2). The expressed BARF0-FLAG protein was detected by immunoblotting with either rabbit 2161 serum or anti-FLAG monoclonal serum. In panel C H1299 cells were transfected with the vector control (lane 1) or the RK-BARF0-FLAG expression vector (lane 2). Rabbit 2161 serum and anti-FLAG monoclonal serum were also used to detect the RK-BARF0-FLAG fusion protein. In panel D radiolabelled BARF0 and RK-BARF0 proteins were in vitro translated, immunoprecipitated with either rabbit 2161 serum or control rabbit serum, and subjected to SDS–12% PAGE. Samples of the direct loads (lane 1) as well as the immunoprecipitates with the rabbit 2161 serum (lane 2) and control serum (lane 3) are shown. kd, kilodaltons.

noprecipitated with either serum from rabbit 2161 or serum from a rabbit immunized with an unrelated peptide (Fig. 1D). As expected from the predicted amino acid sequences of the BARF0 and RK-BARF0 proteins, the in vitro-synthesized proteins migrated on SDS-PAGE gel with estimated molecular masses of approximately 20 (BARF0) and 30 kDa (RK-BARF0). The considerable amount of hemoglobin in the direct load affects the migration of the BARF0 and RK-BARF0 in vitro-translated proteins, which are detected as diffuse bands. However, additional experiments with less protein revealed that only one protein of either 20 or 30 kDa was detected in the direct load. Both BARF0 and RK-BARF0 proteins were immunoprecipitated by serum from rabbit 2161 but

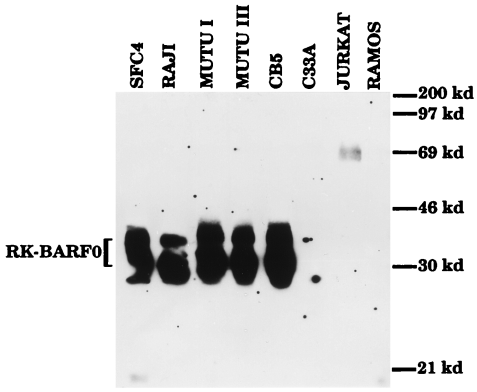

FIG. 2. Detection of RK-BARF0 in cell lines. Samples of five EBV-positive cell lines (SFC4, Raji, Mutu I, Mutu III, and CB5) and three EBV-negative cell lines (C33A, Jurkat and Ramos) were subjected to SDS-PAGE and immunoblotting. RK-BARF0 was detected with anti-BARF0 antiserum. The position of RK-BARF0 is shown as well as the positions of the molecular mass standards. kd, kilodaltons.

not by the unrelated serum, indicating that rabbit 2161 serum contained antibodies specific for the BARF0 ORF product. This serum did not immunoprecipitate other in vitro-translated products, including a 126-aa-encoding ORF present in the previously identified RB2 cDNA and a 103-aa-encoding ORF in the RK1 cDNA, both of which are transcribed in NPC (data not shown) (38).

Identification of RK-BARF0 in EBV-infected cell lines. To determine whether the BARF0 protein was expressed in EBV-transformed cell lines, cell extracts were prepared and analyzed by immunoblotting. SDS extracts were prepared from two EBV-transformed B-cell lines (CB5 and SFC4), three BL cell lines (Raji, Mutu I, and Mutu III), and three EBV-negative epithelial and lymphoid cell lines (C33A, Jurkat, and Ramos). These samples were subjected to electrophoresis on SDS–12% PAGE gel, transferred to nitrocellulose, and immunoblotted with anti-BARF0 serum (Fig. 2). Two proteins with approximate molecular masses of 30 and 35 kDa were detected in the EBV-positive cell lines but not in the EBV-negative lines. Two proteins of the same size were also detected in neonatal lymphocytes 14 days postinfection with B95-8 virus, but they were not detected in the neonatal lymphocytes prior to infection (data not shown). The serum also reacted with proteins at 20 and 69 kDa in some of the cell lines, including the EBV-negative Jurkat cell line. Rabbit antiserum that was affinity purified with Sepharose conjugated with synthetic peptide also reacted specifically with the 30- to 35-kDa proteins, demonstrating that this reaction was not due to nonspecific antibodies in the rabbit serum (data not shown).

These results indicated that two proteins with molecular masses of 30 and 35 kDa are expressed in vivo and were identified with the BARF0-specific antiserum. The molecular masses of the proteins identified in vivo suggest that they represent the 30-kDa RK-BARF0 gene product rather than the 20-kDa BARF0 gene product. The in vitro-translated RK-BARF0 migrates at the same position as the lower 30-kDa band, while the transfected RK-BARF0-FLAG migrates at 35 kDa, the same size as that of the upper band. This suggests that the 35-kDa protein represents a posttranscriptional modification of the 30-kDa RK-BARF0 protein that occurs in vivo in transfected cells but not in the in vitro translations.

Cellular localization of RK-BARF0. To investigate the cellular localization of RK-BARF0, cells were separated into nu-

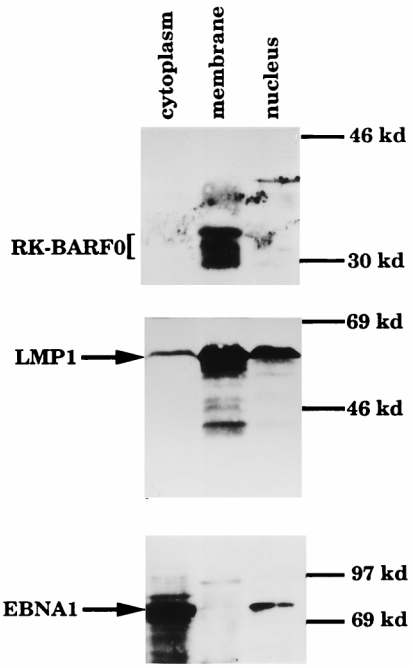

FIG. 3. Cellular localization of RK-BARF0. CB5 cells were separated into nuclear, cytoplasmic, and membrane fractions. Equal protein samples of each fraction were subjected to SDS-PAGE and immunoblotting. Proteins were detected with either the anti-BARF0 antiserum, the anti-LMP1 monoclonal antiserum, or the anti-EBNA1 monoclonal antiserum. The positions of RK-BARF0, LMP1, and EBNA1 are shown. kd, kilodaltons.

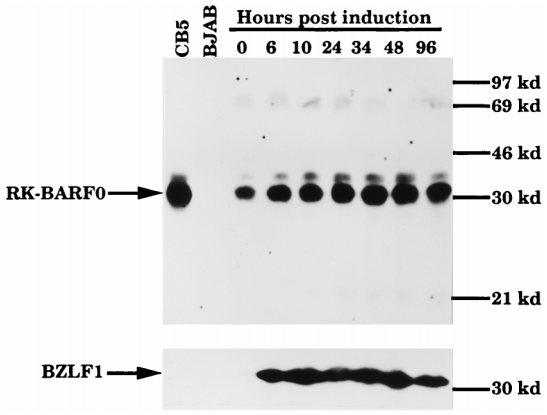

FIG. 4. Detection of RK-BARF0 in Akata cells following induction of EBV replication. Akata cells were induced for EBV virus production with anti-immunoglobulin G. Samples were taken following induction and were subjected to SDS-PAGE and immunoblotting. Evidence of successful induction was determined by detection of BZLF1 expression with anti-BZLF1 antiserum. RK-BARF0 was detected with anti-BARF0 antiserum. The EBV-positive CB5 cell line and the EBV-negative BJAB cell line are included as controls. The positions of BZLF1 and RK-BARF0 are indicated, as are the positions of the molecular mass standards. kd, kilodaltons.

clear, cytoplasmic, and membrane fractions prior to immunoblotting. The EBV-transformed CB5 cell line was used because of the high level of RK-BARF0 protein expressed in these cells (Fig. 2). Samples of the nuclear, cytoplasmic, and membrane fractions were subjected to electrophoresis through SDS–12% PAGE gel, transferred to nitrocellulose, and immunoblotted with either anti-BARF0 serum, anti-EBNA1 monoclonal antibody, or anti-LMP1 monoclonal antibody (Fig. 3). The 73-kDa EBNA1 protein was detected in both the nuclear and cytoplasmic extracts as expected (39), whereas the 63-kDa LMP1 protein was predominantly detected in the membrane fraction. The RK-BARF0 protein was detected only in the membrane preparation, suggesting that the protein is membrane associated. The amino terminus of RK-BARF0 contains a strongly hydrophobic region that may function as an endoplasmic reticulum-targeting signal peptide (38).

Expression of RK-BARF0 after induction of EBV viral replication. To determine if BARF0-related proteins function during replication and to explore the possibility that RK-BARF0 functions in restricting EBV viral replication, as has been suggested for the *Bam*HI A RNA transcripts (22), the expression of RK-BARF0 was analyzed after induction of Akata cells. EBV replication was induced in Akata cells by the addition of anti-immunoglobulin G, and samples were taken at different time intervals up to 96 h postinduction. These cell samples were then subjected to SDS-PAGE and immunoblotting. The degree of induction of EBV was determined with a monoclonal antibody to the EBV replication activator, BZLF1, while the expression of RK-BARF0 was measured with anti-BARF0 serum (Fig. 4). The level of expression of the BZLF1 gene product increased significantly up to 10 h postinduction, after which time its level of expression remained constant, indicating successful induction of EBV replication. RK-BARF0 also increased by 10 h postinduction. The increase in RK-BARF0 indicates that the protein is not involved in restricting replication of EBV. The presence of RK-BARF0 in the CB5 cell line, which is tightly latent as indicated by the lack of BZLF1 expression, implies that RK-BARF0 is a latent protein. However the increase in expression of RK-BARF0 following induction may indicate that the protein functions during both latency and replication. In addition, a 20-kDa protein was detected at low levels approximately 24 h postinduction. This protein may represent the BARF0 protein, suggesting that BARF0 may be expressed during EBV replication.

Expression of RK-BARF0 in EBV-infected tissues. Since the *Bam*HI A transcripts were originally identified in NPC tumor samples where the transcripts are considerably more abundant than in lymphoid cell lines, RK-BARF0 protein expression was analyzed in a number of different tumor biopsies. SDS cell extracts were prepared from six NPC biopsies, including one NPC metastasis, one BL biopsy, and two EBV-negative SCC biopsies. These extracts were then subjected to immunoblot-

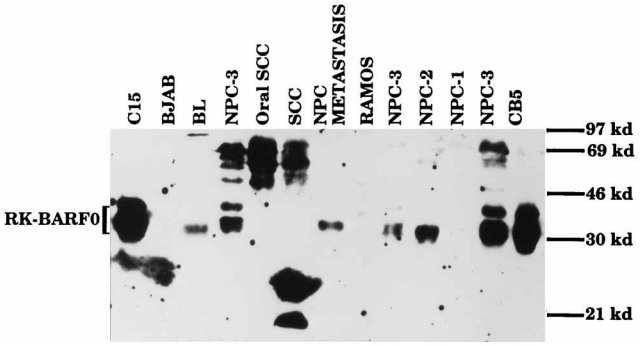

FIG. 5. Immunoblot of tumor biopsies. SDS extracts were prepared from six NPC biopsies (including one metastasis), two SCC biopsies, one BL biopsy, one nude mouse-passaged NPC (C15), two EBV-negative cell lines (Ramos and BJAB), and one EBV-positive cell line (CB5). These extracts were subjected to SDS-PAGE and immunoblotting with anti-BARF0 antiserum. The position of RK-BARF0 as well as those of the molecular mass standards are indicated. The numbers next to the primary NPC biopsies are the WHO classifications.

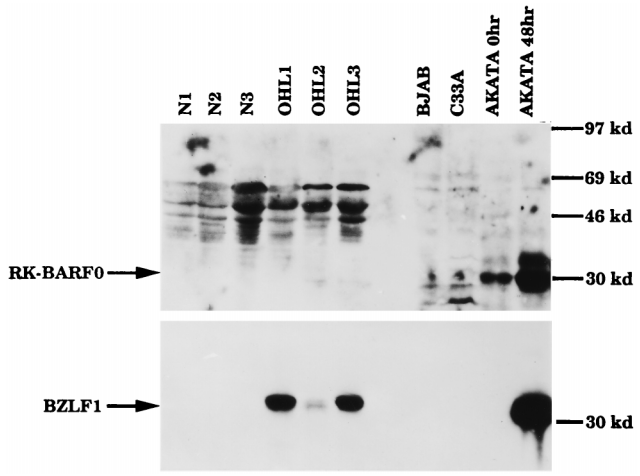

FIG. 6. Immunoblot of OHL lesions. Biopsies of OHL lesions from three HIV-positive patients (OHL1 to OHL3) and lateral tongue border biopsies from three healthy individuals (N1 to N3) were sonicated in SDS buffer. SDS extracts were also prepared from Akata cells (uninduced and induced for 48 h) and from C33A and BJAB, two EBV-negative cell lines. The extracts were subjected to SDS-PAGE and were immunoblotted with either anti-BARF0 or anti-BZLF1 monoclonal antiserum. The positions of RK-BARF0, BZLF1, and the molecular mass standards are indicated. kd, kilodaltons.

ting with the BARF0 antiserum (Fig. 5). The 30- to 35-kDa RK-BARF0 protein was not detected in the EBV-negative B-cell lines Ramos and BJAB or the two EBV-negative SCC samples. RK-BARF0 was detected in the EBV-positive CB5 cell line and in the C15 tumor xenograft as well as in five of the six NPC tumor biopsies. The NPC biopsy in which RK-BARF0 was not detected was a small sample of a WHO 1 NPC sample; it is possible that the sample processed for biochemical analysis lacked sufficient tumor material. In the EBV-positive BL biopsy and two of the NPC samples, only the 30-kDa protein was detected. The apparent absence of the 35-kDa component in these samples could reflect differences in abundance of the proteins or differences in posttranslational processing in some tumor samples.

The AIDS-associated lesion OHL represents a permissive EBV infection characterized by abundant linear EBV DNA, expression of replicative gene products, and lack of expression of the small nuclear EBV-encoded RNAs (EBERs) (15, 18). Oral biopsies were obtained from three HIV-positive individuals with clinically apparent OHL as well as samples of the lateral border of the tongue from three healthy individuals. Immunoblots prepared with extracts from these samples were reacted with anti-BARF0 and anti-BZLF1 antisera (Fig. 6). BZLF1 protein was detected in each of the OHL lesions as well as in Akata cells 48 h after induction of virus production. BZLF1 was not detected in any of the tissue samples from healthy individuals, in uninduced Akata cells, or the two EBV-negative cell lines, BJAB and C33A. RK-BARF0 was not detected in any of the OHL samples or in the biopsies of normal tissue but was detected in both the uninduced and induced Akata cell lines.

Besides detecting RK-BARF0, the rabbit serum also reacted with proteins in the molecular mass range of 46 to 69 kDa. These proteins were present in both NPC biopsy samples and in EBV-negative SCC biopsies, as well as in the biopsies of OHL and normal tissue, and most likely reflect nonspecific reactivity of the antiserum with cellular proteins. These proteins were also detected with the preimmune serum from rabbit 2161, providing additional evidence that these bands are nonspecific (data not shown).

DISCUSSION

The data presented in this study indicate that an RNA transcript from the *Bam*HI A region of EBV encodes a protein that is expressed in vivo. The rabbit serum produced by immunization with an N-terminal BARF0 peptide contains antibodies that are specific for the protein encoded by the BARF0 ORF, as demonstrated by its reactivity on immunoblots to a GST-BARF0 fusion protein, but not to GST alone, and to both the BARF0-FLAG and RK-BARF0-FLAG fusion proteins transfected into H1299 cells. The serum also immunoprecipitates radiolabelled, in vitro-translated BARF0 and RK-BARF0.

The molecular masses of the proteins detected on immunoblots, 30 and 35 kDa, suggest that they most likely represent the RK-BARF0 ORF, which encodes a 30-kDa protein when translated in vitro and whose product is detected as a 35-kDa protein when H1299 cells are transfected with a RK-BARF0-FLAG expression construct. This suggests that the 35-kDa protein detected in transfected cells and tumor lysates likely represents a posttranslational modification of RK-BARF0 that occurs in vivo but not in reticulocyte lysates. The detection of the 30- and 35-kDa proteins in the membrane fraction also suggests that they represent the RK-BARF0 ORF. The RK-BARF0 ORF contains a strongly hydrophobic region that corresponds to an endoplasmic reticulum-targeting signal peptide sequence that is absent from the BARF0 ORF (38), suggesting that RK-BARF0 is a membrane protein.

It has been previously demonstrated that the *Bam*HI A region of EBV is not necessary for transformation of lymphocytes by the virus (35). However, the RK-BARF0 protein was detected in two tightly latent LCLs, CB5 and SFC4, and in neonatal lymphocytes early after infection, suggesting that it functions during latency. At least two other genes, EBNA3B and LMP2, that are consistently expressed in latently infected lymphocytes are also not essential for B-lymphocyte transformation (24, 27–29, 43). That these genes are retained by the virus and consistently expressed in transformed cells suggests that they probably contribute in some way to infection in vivo. RK-BARF0 may also be an important component of infection in vivo.

It is also possible that RK-BARF0 may be essential for latent infection of epithelial cells, which at present cannot be assessed in vitro. *Bam*HI A transcription is significantly higher in NPC than in BL or LCLs, and the relative level of RK-BARF0, as judged by immunoblotting, was higher in the C15 tumor line than in any of the BL or LCLs examined (4, 22). It is of particular interest that RK-BARF0 was consistently detected in NPC, which represents latently infected epithelial cells, but was not found in OHL, a permissively infected epithelial cell lesion. These findings suggest that RK-BARF0 is important in latent epithelial infection but is not essential for viral replication. However, the RK-BARF0 protein was also detected in Akata cells and increased after induction of viral replication, suggesting that it may also function during replication. EBV LMP1, an essential latent protein, also increases during replication, although it is unknown how it contributes to replication (36). The increase in RK-BARF0 protein after induction of replication suggests that it does not restrict viral replication, while the absence of detectable RK-BARF0 in the permissively infected OHL lesions suggests that any replicative function of the protein may be restricted to viral reactivation and replication in B cells.

The 20-kDa protein expressed in Akata cells at low levels

24 h postinduction and in the EBV-positive SFC4 cell line may represent the protein encoded by the BARF0 ORF. This may indicate that the 20-kDa BARF0 protein functions during viral replication. This protein has the same molecular weight as the product of the BARF0 ORF translated in vitro; however, a protein with a similar molecular weight was also detected in the EBV-negative BL cell line, Ramos. Therefore, this protein could represent a cellular protein that is detected because of nonspecific cross-reactivity of the antiserum.

There are several striking differences in EBV expression between latently infected NPC tissues and latently infected, transformed lymphocytes. The EBNA2, -3A, -3B, -3C, and -LP proteins are not detected in NPC but are consistently expressed in B lymphocytes. The functions of all of these proteins are not known; however, EBNA2 and -3C regulate expression of LMP1 and LMP2A and LMP2B (1, 2, 3, 12, 14, 44, 46, 47). It is possible that the *Bam*HI A proteins contribute to viral regulation of gene expression in the absence of the EBNA proteins. The BARF0 ORF encodes three arginine-rich motifs that mark proteins with RNA binding properties (38). This suggests that the BARF0 protein could bind RNA and affect transport and/or processing of viral mRNAs.

The RK-BARF0 protein was also detected in a group I BL cell line (Mutu I) and in a BL biopsy. EBNA1 has been the only viral protein thought to be expressed in BL cells. As yet, cytotoxic T cells specific for EBNA1 have not been identified. EBNA1 is apparently specifically excluded from proteolytic processing and presentation by class I molecules; this property is conferred by a glycine/alanine repeat sequence present in the EBNA1 protein (26). In other viral infections, viral proteins that impair presentation of viral proteins within class I molecules have been identified. It is possible that RK-BARF0 also interferes with this process and enables expression of immunogenic proteins without immune recognition. It will be important to identify more precisely the intracellular locations of the RK-BARF0 protein and interacting cellular proteins to determine the RK-BARF0 protein's molecular properties. However, the detection of RK-BARF0 proteins in both epithelial tumors and B-cell tumors suggests that their expression is an important component of EBV-induced neoplasia.

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