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Posttranslational Processing of an Epstein-Barr Virus-Encoded Membrane Protein Expressed in Cells Transformed by Epstein-Barr Virus

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The *Bam*HI Nhet fragment of the B958 strain of Epstein-Barr virus (EBV) encodes a membrane protein (BNLF-1) that is present in cells transformed by EBV. We made a hybrid protein in which a polypeptide sequence from the carboxyl-terminal part of BNLF-1 is fused to *Escherichia coli* β-galactosidase. This hybrid protein was used to immunize rabbits, and the resulting antiserum was purified by immunoaffinity chromatography. The antiserum was able to immunoprecipitate BNLF-1 from cell lysates. We found that BNLF-1 is phosphorylated at serines in EBV genome-positive B-cell lines. Pulse-chase analyses with [^{35}S]methionine indicated that BNLF-1 is turned over in lymphoblasts with a half-life of approximately 5 h. Protein immunoblots of EBV genome-positive B-cell lines revealed both a 62,000-molecular-mass band corresponding to BNLF-1 and a myriad of lower-molecular-mass bands. We postulate that these lower-molecular-mass bands are degradation products resulting from the turnover of BNLF-1 in cells. The BNLF-1 gene was expressed in COS cells, and the protein was both phosphorylated and turned over in these cells.

Epstein-Barr virus (EBV) is a lymphotropic herpesvirus associated with at least two human malignancies, African Burkitt's lymphoma and nasopharyngeal carcinoma (reviewed in reference 23). In vitro, the virus can infect and immortalize nondividing B-lymphocytes into permanently proliferating B-lymphoblasts (reviewed in reference 23). In EBV-immortalized B-lymphoblasts, the productive cycle of the virus occurs infrequently (less than 1 cell per 10^4 per cell generation), and the viral infection is considered latent (31).

Viral DNA sequences needed for initiation and maintenance of the transformed state of B-cells have not been identified. However, when appropriately expressed, a gene encoded by the *Bam*HI Nhet fragment of the genome (BNLF-1) has been shown to alter the growth properties of an established rodent cell line (Rat-1) (32). (We refer to the protein product formed by the fusion of the BNLF-1a, BNLF-1b, and BNLF-1c open reading frames [1] as the BNLF-1 protein. The gene encoding this protein is referred to as the BNLF-1 gene. This gene has been referred to previously as the LYDMA, LMP, or p63 gene [11, 22, 32]. The protein product encoded by the message from the ED-L1A promoter [2], which is present in cells late in lytic infection, is referred to as the lytic BNLF-1 protein.) Expression of the BNLF-1 gene leads to loss of contact inhibition, anchorage-independent growth in soft agar, and tumorigenic transformation of Rat-1 cells. Based on these observations, it has been suggested that the BNLF-1 gene might have a role in the transformation of B-lymphocytes by EBV. However, no biochemical activities have been associated with the BNLF-1 protein in transformed cells.

The BNLF-1 protein was first postulated to be present in transformed lymphoblastoid cells because the *Bam*HI Nhet region of the viral genome was found to encode a 2.8-kilobase mRNA in these cells (7, 12). A 58,000- to 63,000-molecular-mass protein (58K to 63K protein) has been identified by protein immunoblots in cell lysates of transformed cells with antibodies made to a part of BNLF-1 (11, 22). Both immunofluorescence analysis and biochemical fractionation of cell lysates followed by protein immunoblots indicate that a significant portion of the protein is present in the plasma membrane of cells (11, 22). A secondary structure has been proposed for BNLF-1 in which the polypeptide chain crosses the membrane six times such that both the amino and carboxyl termini of the protein are cytoplasmic (2, 7). Experiments that determine the sites in the protein that are accessible to proteolytic cleavage in live cells have been used as evidence in support of the proposed secondary structure (20).

As a step toward the functional characterization of the BNLF-1 protein in transformed lymphoblastoid cells, we generated a polyclonal antiserum directed to a 166-amino-acid polypeptide fragment from the carboxyl-terminal portion of the protein and used it as a reagent to characterize BNLF-1 biochemically. With this antiserum, we detected a protein of approximately 62K in immunoprecipitates of [^{35}S]Met-labeled EBV genome-positive lymphoblastoid cells. We also detected an additional protein of approximately 43K in virus-producing cells treated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA), as predicted previously (12). The BNLF-1 protein is phosphorylated at serines and has a metabolic half-life of approximately 5 h in EBV-transformed cells. In protein immunoblots of lysates from EBV-genome positive cells, we found a ladder of bands that extends up to approximately 62 kilodaltons, which is consistent with the degradation of BNLF-1 in these cells. Since BNLF-1 is a membrane protein, the observed turnover of BNLF-1 in EBV genome-positive cells might represent endocytosis and lysosomal degradation.

MATERIALS AND METHODS

Cells. *Escherichia coli* JS1060 (33) was used as a host for transformation in constructing the plasmid 292.1. JS1060 containing the plasmid 292.1 [JS1060(292.1)] was used as the source for purifying the fusion protein, BNLF-1–β-galacto-

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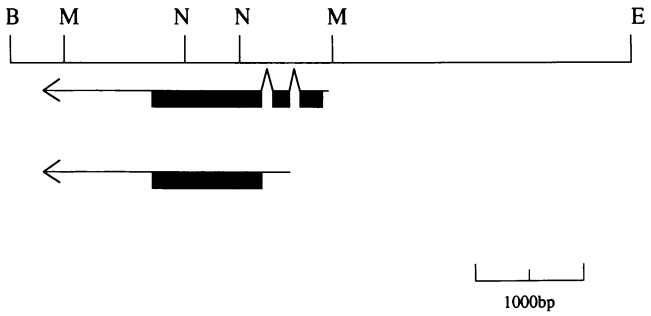

FIG. 1. Restriction enzyme cleavage site map of a portion of the *Bam*HI Nhet fragment of the B958 strain of EBV. The region shown is from the *Bam*HI site at nucleotide 166616 to the right-hand terminus at nucleotide 172282 (1). The restriction enzyme sites that are indicated are *Bam*HI (B), *Eco*RI (E), *Mlu*I (M), and *Nco*I (N). The doubly spliced mRNA represents the 2.8-kilobase mRNA that is found in cells transformed by EBV (7, 12). The filled boxes under this mRNA represent the coding sequences for the BNLF-1 protein. The unspliced shorter mRNA (2.5 kilobases) represents the mRNA found in TPA-treated B958 cells late in the lytic cycle of the virus. The filled box under this message represents the coding sequences for the lytic BNLF-1 protein. The coding sequences of the lytic BNLF-1 protein are a subset of the coding sequences of the BNLF-1 protein that is found in latently infected cells, since both proteins are encoded in the same reading frame (2).

sidase. *E. coli* DH1 was used for all other plasmid constructions. Cells were routinely grown in Luria broth. Ampicillin (final concentration of 50 μg/ml) was used to select cells containing plasmids.

3/15-31 and 721 are B-cell lines established by transformation of human B-lymphocytes in vitro (14, 31) with EBV. Raji is an EBV genome-positive Burkitt's lymphoma cell line (26). B958 is a marmoset lymphoblastoid cell line established by transformation in vitro (24). IA3, a clone of B958, was used in all experiments. IA3 cultures spontaneously release virus, and 1 to 5% of the cells are positive for the presence of the viral capsid antigen as judged by indirect immunofluorescence. Manca, Wilson, Ramos, and BJAB are EBV genome-negative Burkitt's lymphoma cell lines (15, 16). COSM6 is a cell clone of the COS cell line and expresses simian virus 40 (SV40) T antigen (8). All lymphoblastoid cell lines, except B958, were grown in RPMI 1640 plus 10% fetal bovine serum. B958 cells were grown in RPMI 1640 plus 5% calf serum. COS cells were grown in Dulbecco high glucose plus 10% fetal bovine serum. TPA (20 ng/ml) was used to induce the viral lytic cycle in IA3 cultures where indicated. All cells were grown at 37°C in a 5% CO_2 atmosphere.

Plasmids. pJS413 is an expression vector which has unique restriction enzyme sites for introducing foreign DNA fragments at the N terminus of the *lacI-Z* gene (33). A 499-base-pair *Nco*I fragment containing sequences coding for the carboxyl-terminal part of BNLF-1 was repaired with the Klenow fragment of *E. coli* DNA polymerase I (Fig. 1). The blunt-ended *Nco*I fragment was ligated with *Sma*I-digested pJS413 to obtain the plasmid 292.1. In p292.1, the reading frame for BNLF-1 is in the same orientation and phase as the β-galactosidase reading frame. pHK412 is similar to pJS413, except that it encodes an uninterrupted β-galactosidase protein. pSV_2BNLF-1 was constructed by replacing the blunt-ended *Hin*dIII-to-*Hpa*I fragment containing the CAT gene from pSV_2CAT (9) with a blunt-ended *Mlu*I fragment containing BNLF-1 (Fig. 1), such that the SV40 enhancer is upstream of the BNLF-1 coding sequences. p264.2 contains the SV40-early-promoter–β-galactosidase fusion from plasmid CH110 (10) inserted into plasmid 220.2. p220.2 is similar to p201, except that it contains a polylinker (35).

Isolation of fusion protein. An overnight culture of JS1060(292.1) was diluted 1:20 in Luria broth containing 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and cells were allowed to grow for 6 to 7 h with vigorous shaking. Cells were then collected by centrifugation and stored frozen at −20°C before use. Cells were lysed by thawing in three parts (by weight) of buffer A containing 40 mM NaCl (buffer A is 20 mM Tris hydrochloride, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, 2% Trasylol[FBA Pharmaceuticals], pH 7.4), followed by two to four passages through a French press at 10,000 lb/in^2. The cell lysate was clarified by centrifugation at 100,000 × *g* for 1 h at 4°C. The supernatant was brought to 25% saturation of $(NH_4)_2SO_4$ by gradually adding a solution of 100% saturated $(NH_4)_2SO_4$. The suspension was stirred at 4°C for 30 to 45 min. The precipitated material was removed by centrifugation at 1,200 × *g*. The supernatant was then brought to 40% saturation of $(NH_4)_2SO_4$ as before. The pellet was collected by centrifugation as before and resuspended in a minimal volume of buffer A containing 200 mM NaCl. The solution was then dialyzed against several changes of the same buffer (minus Trasylol), made to 10% in glycerol, clarified by centrifugation, and fractionated on a Sephacryl S-300 sizing column (90 by 2.6 cm diameter). Fractions containing the fusion protein were identified by assaying for β-galactosidase (25) and analyzing samples by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fractions containing the fusion protein were pooled and concentrated by ammonium sulfate precipitation. All procedures, except chromatography on the S-300 column, were done at 4°C. The S-300 column was run at room temperature. The purified material was greater than 95% pure as judged by Coomassie blue staining of electrophoresis gels (data not shown). The purity is probably underestimated, since the fusion protein was found to be susceptible to proteolysis. The β-galactosidase used for all experiments was purified in a similar manner from JS1060 cells containing pHK412.

Immunization of rabbits. New Zealand White female rabbits were immunized by monthly injections of approximately 200 μg of purified fusion protein emulsified with Freund incomplete adjuvant, after the initial immunization in Freund complete adjuvant. The animals were bled at 7 to 10 days postimmunization, and the serum was stored frozen. Enzyme-linked immunosorbent assays (ELISA) indicate that the titer of the antiserum steadily increased to greater than $1:10^6$ after 12 immunizations. Antiserum with an approximate titer of $1:10^4$ was used for these experiments.

Purification of the antiserum. Two affinity matrices were prepared for this purpose. Partially purified β-galactosidase (100 mg; approximately 70% pure) was bound sequentially to Affi-gel 15 and Affi-gel 10 (Bio-Rad Laboratories) to obtain a mixed column of approximately 4 ml (column A). Purified BNLF-1–β-galactosidase fusion protein (20 mg) was bound to Affi-gel 15 to obtain column B. The instructions of the manufacturer (Bio-Rad) were followed for binding the proteins to the column and running the column. For both columns, greater than 90% of the protein was bound to the column, as judged by monitoring the unbound effluent by A_{280}. To purify a batch of antiserum, approximately 20 ml of antiserum was passed first over column A to remove antibody molecules directed against the β-galactosidase part of the fusion protein. The flowthrough from column A was pooled and passed over column B. Column B was then

washed extensively, sequentially with phosphate-buffered saline (PBS) and PBS–1.0 M NaCl until the eluate had an A_{280} of less than 0.05. The bound material was then eluted with buffer B (10 mM Tris, 1 M KSCN, pH 9). Fractions of the eluted material were identified by readings of A_{280}, pooled, dialyzed versus PBS, and then passed a second time over column A to remove any residual activity bound by β-galactosidase. Column A was regenerated by washing the column with buffer B. The flowthrough from the second passage over column A was pooled and represents the immunoaffinity purified antiserum used for all experiments. The purification of the antiserum was monitored by comparing the titer obtained in ELISA with β-galactosidase with that obtained with the fusion protein. The titer for reactivity with the fusion protein remained at $1:10^4$ through the purification, whereas the reactivity with β-galactosidase dropped from a titer of $1:10^4$ to less than 1:10. Moreover, the purified antiserum detected the fusion protein on a protein immunoblot, but showed no reactivity with an equal amount of purified β-galactosidase (data not shown).

Enzyme-linked immunosorbent assays. Enzyme-linked immunosorbent assays were done as previously described (6). Briefly, wells of a 96-well plate were coated with antigen by incubating the plate containing antigen (5 μg/ml in coupling buffer) overnight at 4°C. Antibody molecules bound to the antigen were detected with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G. Color development, after incubation with the substrate *p*-nitrophenyl phosphate, was monitored with a Titertek Multiskan plate reader (Flow Laboratories, Inc.).

Protein immunoblots. Frozen cell pellets were lysed by suspending the cells at 10^8 cells per ml for nonadherent cells and 10^7 cells per ml for adherent cells in 1% SDS–1% Trasylol in sterile water. Lysates were sonicated (two times, 15 s each) to shear DNA. Cell lysates were kept on ice and used within an hour of preparation. Generally, cell lysates corresponding to 10^5 nonadherent cells or 10^4 adherent cells were resolved on a 10% SDS-polyacrylamide gel. Proteins were transferred electrophoretically from the gel onto a nitrocellulose paper as described previously (21). Filters were incubated with PBS containing 1% Carnation instant nonfat dry milk to reduce background. The immunoaffinity-purified antiserum was used at a final dilution of 1:50 or 1:100. Alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G was used as a detecting antibody at a dilution of 1:500 or 1:1,000 (Sigma Chemical Co.). Incubations were for approximately 1 h at 37°C, and blots were washed after the incubations, with PBS–0.05% Tween 20. Color development was done as described previously, by using a combination of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (19).

Radiolabeling of cells and immunoprecipitation. For lymphoblastoid cells, approximately 10^7 exponentially growing cells were washed once with medium A (RPMI 1640 minus methionine plus 10% dialyzed fetal bovine serum) and then incubated with 1 ml of the same medium containing 500 μCi of [^{35}S]Met (specific activity, 1,150 Ci/mmol) for 4 to 5 h. For COS cells, a confluent 100-mm-diameter dish was rinsed twice with medium A and then incubated with 2 ml of the same medium containing 500 μCi of [^{35}S]Met. For ^{32}P labeling, the labeling medium used was RPMI 1640 minus phosphate plus 10% dialyzed fetal bovine serum, and the radioisotope used was [^{32}P]orthophosphoric acid (1 mCi; specific activity, 6,000 Ci/mmol). For all labelings, cells were incubated in medium minus radioisotope for 30 min before addition of the radioisotope. At the end of the labeling period, lymphoblastoid cells were collected by centrifugation, washed once with PBS, and then stored frozen at −20°C. COS cells were detached from the plate by trypsinization and then collected by centrifugation. Frozen cells were thawed and lysed by addition of 5× RIPA buffer (1× RIPA buffer is 0.15 M NaCl, 1% [vol/vol] Triton X-100, 0.5% [wt/vol] sodium deoxycholate, 0.5% [wt/vol] SDS, 1% [vol/vol] Trasylol, and 0.01 M Tris, pH 7.2) and occasional vortexing. The lysate was made 1× in RIPA buffer by the addition of sterile water and was then heat treated for 10 min at 85°C, before being used for immunoprecipitation. Immunoprecipitation was done essentially as described (30). Lysates were precleared by sequential incubation with affinity-purified rabbit anti-human $(Fab')_2$ antibodies, goat anti-rabbit immunoglobulin, and formaldehyde-fixed *Staphylococcus aureus* cells. The suspension was clarified by centrifugation at 1,200 × *g* for 45 min. Generally, the supernatant was divided equally into two parts and incubated for 30 min either with affinity-purified antiserum (equivalent to 25 μl of unpurified serum) or with 50 μl of normal rabbit serum. Antigens were precipitated by sequential incubation with goat anti-rabbit antiserum and formaldehyde-fixed *S. aureus*. Pellets were collected by centrifugation in a Microfuge (Beckman Instruments, Inc.) for 5 min, washed five times in 1× RIPA buffer, and then boiled with sample buffer (18) for 10 min. The supernatant was resolved in a 10% acrylamide gel (18). The gel was then impregnated with En3Hance (New England Nuclear Corp.) as recommended by the manufacturer, dried, and exposed to an Eastman Kodak XAR-5 film at −70°C. Generally, fluorographic signals were visible after an overnight exposure.

In control experiments, the amount of BNLF-1 protein retained in the preclearing pellet during immunoprecipitation was determined by solubilizing the pellet in SDS-PAGE sample buffer (18), resolving the suspension by SDS-PAGE, and detecting BNLF-1 by protein immunoblots. With such an analysis, no greater than 20 to 25% of the starting material was recovered in the pellet (data not shown). If cells were first fractionated (0.5% Triton X-100 in PBS; centrifugation conditions, 100 × *g*, 10 min, 0°C) into a Triton X-100-soluble (approximately 85% of the total BNLF-1) or Triton X-100-insoluble fraction (approximately 15% of the total BNLF-1) and each fraction was analyzed separately as above, then up to 75% of the Triton X-100-insoluble fraction could be solubilized with 1× RIPA buffer. This observation indicates that the BNLF-1 protein that is lost in the preclearing precipitation does not arise solely from a Triton X-100-insoluble fraction of the cell but more likely represents a nonspecific loss.

SDS-PAGE analysis. Gels (10% acrylamide) were used to separate proteins (18). Prestained high-molecular-mass markers (Bethesda Research Laboratories) were used on all gels as molecular-mass standards. The markers were myosin heavy chain, 200,000; phosphorylase *b*, 97,400; bovine serum albumin, 68,000; ovalbumin, 43,000; and α-chymotrypsinogen, 25,700 molecular mass.

Two-dimensional phosphoamino acid analysis. Two-dimensional phosphoamino acid analysis was done as described previously (13). Immunoprecipitates of lysates from ^{32}P-labeled cells were separated on gels, and autoradiograms of the gel were used to cut out portions of the gels containing the desired proteins. Proteins were eluted as described previously (3) and subjected to phosphoamino acid analysis. Unlabeled phosphoamino acids (P-Ser, P-Thr, and P-Tyr) were used as standards for the electrophoresis and visualized by staining with ninhydrin.

Electroporation. Approximately 1.5×10^7 COS cells were electroporated with 5 to 10 μg of plasmid DNA as described previously (29). Cells were then plated at approximately 10^6 cells per 100-mm-diameter dish.

RESULTS

Antiserum immunoprecipitates BNLF-1 from COS cells. Antibodies directed against BNLF-1 have been described in the literature (11, 22). However, none of the reports describes immunoprecipitation of BNLF-1 from cell lysates. We determined whether the antiserum we obtained could immunoprecipitate BNLF-1 from cell lysates, since having an antiserum that can immunoprecipitate BNLF-1 from cells would permit additional biochemical characterization of the protein.

$pSV_2BNLF-1$ was constructed to facilitate expression of BNLF-1 in COS cells. In $pSV_2BNLF-1$, the entire coding sequences for BNLF-1 are downstream from the SV40 enhancer and early promoter. The plasmid also contains a functional origin of replication for SV40 and is therefore expected to replicate in COS cells, which express the SV40 T antigen (8). COS cells electroporated with $pSV_2BNLF-1$ were labeled metabolically with [^{35}S]Met or [^{32}P]orthophosphoric acid at 4 to 5 days postelectroporation. Cell lysates were incubated either with normal rabbit serum or with immune serum, and formaldehyde-fixed *S. aureus*-mediated precipitation was used to separate the antigen-antibody complexes. The precipitates were analyzed by SDS-PAGE. Figure 2A shows a fluorogram of such a gel. The immune serum precipitated a protein of approximately 62K as well as proteins of lower molecular mass from both ^{35}S- and ^{32}P-labeled lysates (Fig. 2A). The precipitation of these proteins was blocked by preincubating the antiserum with the fusion protein but not by preincubating the antiserum with β-galactosidase (Fig. 2B). The molecular mass observed for BNLF-1 (approximately 62,000 daltons) is in close agreement with the mass previously reported for this protein (58,000 to 63,000 daltons) (11, 22). (We did not attempt to determine precisely the apparent molecular mobility of the BNLF-1 protein on SDS-PAGE. The protein has an apparent molecular mass of 58,000 to 62,000, depending on the conditions used for SDS-PAGE. For the sake of convenience, we ascribe a molecular mass of 62,000 daltons to the BNLF-1 protein. All other assignments of molecular mass are also approximate.) We do not know the origin of the lower-molecular-mass bands seen in the ^{35}S immunoprecipitates. However, for the following reasons we presume they represent proteolytic degradation products generated in vivo: (i) immunoprecipitates of COS cells electroporated with pSV_2CAT (a plasmid similar to $pSV_2BNLF-1$ except that it contains coding sequences for the chloramphenicol acetyltransferase gene instead of BNLF-1) showed no such proteins, and (ii) pulse-chase experiments of COS cells electroporated with $pSV_2BNLF-1$ indicated that the 62K protein is turned over with a half-life of approximately 2 h, and the label is chased from the 62K protein into proteins with lower molecular mass (data not shown). The approximately 43K protein seen in ^{32}P-labeled cells might be a degradation product also or might represent the lytic BNLF-1 protein (see below).

Immunoprecipitation of BNLF-1 from B-cell lines labeled with [^{35}S]Met. To detect BNLF-1 in B-cell lines, cells were pulse-labeled with [^{35}S]Met for approximately 5 h, and cell lysates were immunoprecipitated with normal serum or immune serum. EBV genome-positive lymphoblastoid (721), Burkitt's lymphoma (Raji), or producer (B958) cell lines revealed a 60K to 62K protein that is precipitated with immune serum but not normal rabbit serum (Fig. 3A). The two EBV genome-negative Burkitt's lymphoma cell lines showed no specific precipitation of proteins with immune serum. These observations indicate that the protein precipitated specifically in EBV genome-positive cells is BNLF-1. The prominent band seen at approximately 75 kilodaltons in Ramos and Raji cells, or approximately 55 kilodaltons in 721 cells, probably represents the immunoglobulin heavy chain molecule synthesized by the cells, which is a contaminant in the immunoprecipitate. The size of BNLF-1 in Raji and in 721 cells is different (Fig. 3B). The observation that different EBV genome-positive cell lines have different-size BNLF-1 proteins, as detected in protein immunoblots, has been reported (11, 22).

On the basis of the structure of the RNAs encoded by this region of the genome in producer (B958) cells treated with TPA, a treatment which enhances the viral lytic cycle, it has been predicted that a protein corresponding to the carboxyl two-thirds of the BNLF-1 protein is synthesized in cells during the lytic cycle of the virus (see Fig. 1) (12). Our

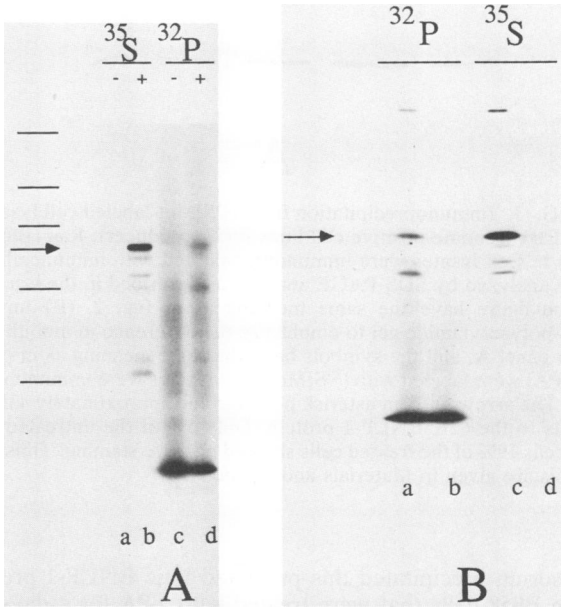

FIG. 2. Immunoprecipitation from cell lysates of COS cells electroporated with $pSV_2BNLF-1$. (A) COS cells electroporated with $pSV_2BNLF-1$ were labeled metabolically 4 to 5 days postelectroporation with [^{35}S]Met (^{35}S) or [^{32}P]orthophosphoric acid (^{32}P) for 4 to 5 h. Cell lysates were immunoprecipitated with normal rabbit serum (−, lanes a and c) or immunoaffinity-purified antiserum (+, lanes b and d). Immunoprecipitates were analyzed on a 10% SDS-polyacrylamide gel. The proteins separated on the gel were visualized by fluorography. The arrow to the left indicates the size of the intact BNLF-1 protein. The lines to the left indicate the position of the molecular-mass markers run on the gel. The markers, listed in the Materials and Methods section, have masses of 200, 97.4, 68, 43, and 25.7 kilodaltons. (B) COS cells electroporated with $pSV_2BNLF-1$ were labeled as in panel A with [^{35}S]Met (^{35}S) or [^{32}P]orthophosphoric acid (^{32}P). Cell lysates were immunoprecipitated with immunoaffinity-purified antiserum that was preincubated for 30 min at 4°C with 60 μg of β-galactosidase (lanes a and c) or 60 μg of fusion protein (lanes b and d). Immunoprecipitates were analyzed as in panel A. The arrow to the left indicates the size of the intact BNLF-1 protein. For panels A and B, details of the experimental procedures are given in Materials and Methods.

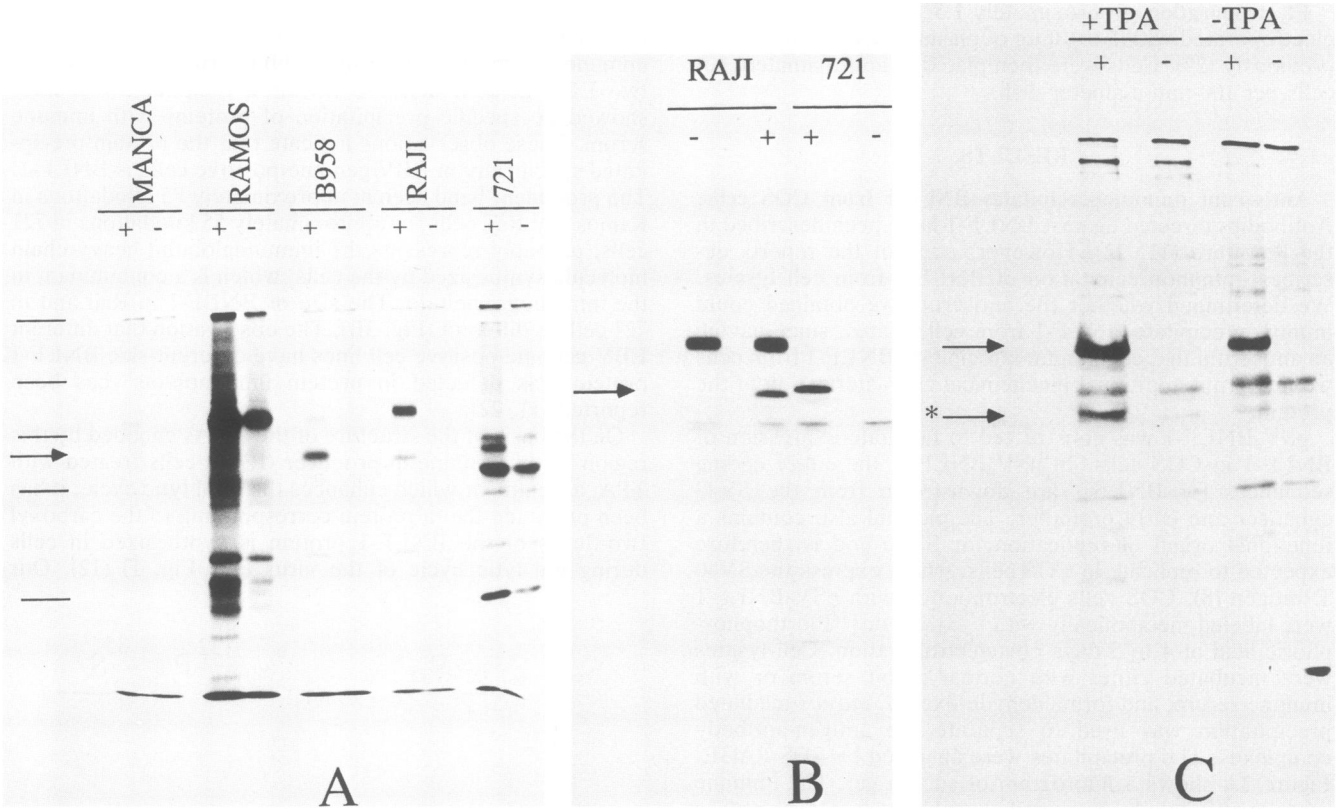

FIG. 3. Immunoprecipitation from [^{35}S]Met-labeled cell lysates from B-cell lines. (A) EBV genome-negative cell lines Manca and Ramos and EBV genome-positive cell lines B958 (producer), Raji (Burkitt's lymphoma), and 721 (in vitro transformed) were labeled with [^{35}S]Met for 4 h. Cell lysates were immunoprecipitated with immunoaffinity-purified antiserum (+) or normal rabbit serum (−). Immunoprecipitates were analyzed by SDS-PAGE analysis as described in the legend to Fig. 2 and detailed in Materials and Methods. The markings on the left of the figure have the same meaning as in Fig. 2. (B) Immunoprecipitates from Raji and 721 cells run in neighboring lanes of an SDS-polyacrylamide gel to emphasize the difference in mobility of BNLF-1 from the two cell lines. The immunoprecipitates were analyzed as in panel A, and the symbols have the same meaning as in panel A. (C) B958 cells treated with TPA (+TPA) for 4 days or mock treated (−TPA) were labeled with [^{35}S]Met. Cell lysates were immunoprecipitated with immunoaffinity-purified antiserum (+) or normal rabbit serum (−). The arrow with an asterisk points to the approximately 43K lytic BNLF-1 protein seen in B958 cells treated with TPA. The other arrow points to the 62K BNLF-1 protein. Only 5% of the untreated cells were stained by indirect immunofluorescence for viral capsid antigen, whereas 19% of the treated cells showed positive staining. Thus, TPA treatment did enhance the number of cells releasing virus. Experimental details are given in Materials and Methods.

antiserum precipitated this predicted lytic BNLF-1 protein from B958 cells that were treated with TPA for 4 days. In B958 cells treated with TPA, an additional protein of approximately 43,000 molecular mass was precipitated by the immune serum but not by the normal serum (Fig. 3C). This additional band is also seen in protein immunoblots with immune serum (data not shown). The difference in size between BNLF-1 and the 43K protein is as expected, based on the structure of the lytic mRNA (12). The amount of the 62K protein synthesized in TPA-treated cells did not differ significantly from that synthesized in mock-treated cells (Fig. 3C), even though the amount of viral DNA increases by approximately 10-fold with such a treatment. This lack of correlation between the amount of a viral protein and the number of copies of a viral genome has been observed also with EBNA-1, which is another viral protein that is expressed in latently infected cells (34).

BNLF-1 is phosphorylated in EBV genome-positive cells. BNLF-1 was phosphorylated in COS cells (Fig. 2). To determine whether BNLF-1 is phosphorylated in EBV genome-positive cells, 721 cells were labeled with $^{32}P_i$ for 4 to 5 h, and cell lysates were precipitated with immune serum or normal rabbit serum. Precipitation with immune serum, but not normal serum, revealed two prominent bands (Fig. 4A). The larger protein is approximately 62K, and the smaller protein is 23K. Precipitation of both proteins was blocked by preincubating the antiserum with fusion protein but not by preincubating the antiserum with β-galactosidase (data not shown). Immunoprecipitation of ^{32}P-labeled lysates from Raji revealed a protein of approximately 62K, and the protein from Raji migrated faster than the protein from 721 cells (Fig. 4B). The 62K phosphoprotein from 721 cells had a V8 protease cleavage map that was similar, albeit not identical, to that of the 62K protein obtained from ^{35}S-labeled lysates of 721 cells (data not shown). All these observations indicate that the 62K protein shown in Fig. 4 represents a phosphorylated form of BNLF-1. In addition, immunoprecipitates from B958 cells treated with TPA contain two phosphoproteins (approximately 62K and 43K), indicating that the lytic BNLF-1 is also phosphorylated (data not shown).

We do not know the origin of the 23K phosphoprotein in

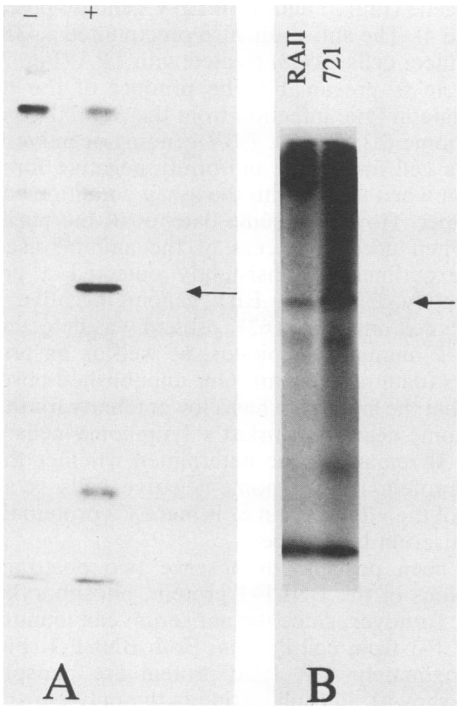

FIG. 4. Immunoprecipitation from cell lysates of B-cell lines labeled with [^{32}P]orthophosphoric acid. (A) 721 cells were labeled with [^{32}P]orthophosphoric acid for 4 h. Cell lysates were immunoprecipitated with normal rabbit serum (−) or with immunoaffinity-purified antiserum (+). Precipitates were analyzed as described in the legend to Fig. 2. The arrow points to the approximately 62K phosphoprotein. (B) Mobility of the approximately 62K phosphoprotein from Raji cells is different from the mobility of the approximately 62K phosphoprotein from 721 cells. Both cell lines, labeled as in panel A, were immunoprecipitated with immunoaffinity-purified antiserum. Precipitates were analyzed as described in the legend to Fig. 2. The arrow points to the approximately 62K phosphoprotein from these cells.

721 cell lysates, but it seems likely to be a proteolytic product of the 62K protein. This protein is always detected in 721 and B958 cell lysates (data not shown) but only occasionally in Raji cell lysates.

To determine the phosphoamino acids associated with BNLF-1, the 62K phosphoprotein immunoprecipitated from 721 cells was isolated by eluting it from a preparative gel, and it was subjected to two-dimensional phosphoamino acid analysis. Figure 5 is a fluorogram of the thin-layer chromatography plate that was used to separate the acid-hydrolyzed protein, and it indicates that phosphoserine is associated with the protein. It is possible that there are other phosphoamino acids associated with BNLF-1 that might not have been detected, since the recovery of ^{32}P counts in the phosphoamino acid spot is not quantitative.

BNLF-1 is turned over in cells. We used a pulse-chase analysis to determine whether BNLF-1 is turned over in cells. 721 cells were labeled metabolically with [^{35}S]Met for 4 h and chased for up to 26.5 h. The amount of [^{35}S]Met incorporated into TCA-insoluble material at 26.5 h postchase is 37% of that incorporated at the start of the chase, indicating that the average half-life for total cellular protein is much longer than 5 h (Fig. 6). The intensity of the signal corresponding to BNLF-1 decreased with a half-life of approximately 5 h as determined by scanning multiple fluorographic exposures of the gel (Fig. 6) with a laser densitometer. Thus the half-life of BNLF-1 (approximately 5 h) is considerably shorter than the average half-life of the total cellular protein.

Figure 7A shows the results of a protein immunoblot. Cell lysates separated by SDS-PAGE were electrophoretically transferred to nitrocellulose, and the nitrocellulose was then incubated with immune serum. As expected, the antiserum detected proteins in the genome-positive cell lines 3/15-31, 721, and Raji but not in the genome-negative Manca, Wilson, and BJAB. Furthermore, the slowest-migrating band in Raji was faster than the slowest-migrating band in 721. This difference in mobility is similar to that observed with ^{35}S immunoprecipitates from these cell lines (Fig. 3B). Interestingly, all three genome-positive cell lines showed additional bands of lower molecular mass. Lysates from COS cells electroporated with pSV_2BNLF-1 showed a similar ladder of bands, whereas control cells electroporated with a plasmid containing β-galactosidase sequences showed no bands. It is striking that bands corresponding to the lower-molecular-mass proteins from the two lymphoblastoid cell lines 721 and 3/15-31 comigrated and, moreover, had the same mobility as the bands in COS cells (Fig. 7A).

The bands in 721 were not detected when the antiserum was preincubated with the fusion protein (Fig. 7B). However, preincubation of the antiserum with β-galactosidase did not prevent detection of the bands in 721 cells. This observation indicates that the epitopes that are being recognized in the lower-molecular-mass proteins are epitopes that are present in BNLF-1. Therefore, it seems likely that this ladder of bands represents the accumulation of metastable degradation products of BNLF-1 resulting from its intracel-

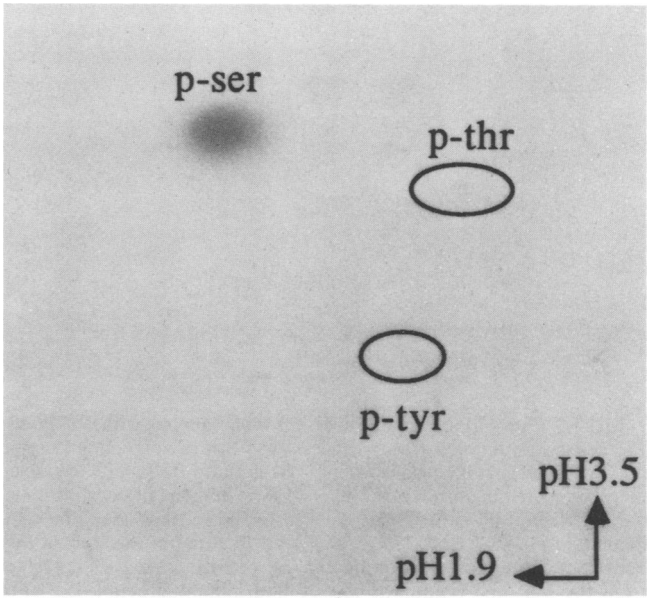

FIG. 5. Phosphoamino acid analysis of the 62K phosphoprotein from 721 cells. Cell lysates from approximately 3×10^7 cells labeled with 2.5 mCi of [^{32}P]orthophosphoric acid were immunoprecipitated with immunoaffinity-purified antiserum. The precipitated protein was eluted from the gel (3), acid hydrolyzed, and resolved electrophoretically (13). The chromatogram was then exposed to an X-ray film. Unlabeled P-ser, P-thr, and P-tyr were used as standards. The positions of the unlabeled P-thr and P-tyr are shown schematically. The orgin for the chromatogram is not shown; it would be beyond the bottom right.

lular turnover. The ladder of bands probably does not represent proteolysis in vitro in cell lysates, since in control experiments incubation of lysates up to 5 h at 37°C did not result in a significant change in the intensity of the largest-molecular-mass protein band from 721 cells (data not shown).

DISCUSSION

The *Bam*HI Nhet fragment of the EBV genome is a region of the genome that is transcribed in cells transformed by EBV (7, 12). The gene product (BNLF-1) encoded by this transcribed region is a membrane protein (11, 22). Rabbit antiserum and murine monoclonal antibodies have been used to demonstrate that BNLF-1 is present in all EBV genome-positive cells that have been examined (11, 22). Expression of BNLF-1 in Rat-1 cells changes their growth properties, and the cells are transformed (32). However, no biochemical activities have been associated with BNLF-1, and the molecular basis for its transforming ability is unknown.

We generated an immunoaffinity-purified antiserum to 166 amino acids from the carboxyl-terminal portion of BNLF-1. It precipitated BNLF-1 from COS cells that contain the BNLF-1 gene (Fig. 2) and from EBV genome-positive cells (Fig. 3 and 4). The antiserum also precipitated a 43K protein from producer cells (B958) treated with TPA (Fig. 3C). This 43K protein is presumably the product of the transcript encoded late in lytic infection from the *Bam*HI Nhet region of the genome (12; Fig. 1). EBV genome-negative Burkitt's lymphoma cell lines were uniformly negative for BNLF-1 expression when tested with the assay conditions described in this paper. However, some batches of the purified antiserum, when used in excess of the amount used in the present experiments, consistently detected a protein of approximately 62K in the EBV genome-negative Burkitt's lymphoma cell lines. This 62K protein was detected in both ^{35}S and ^{32}P immunoprecipitates, as well as on protein immunoblots (data not shown). Our unpublished observations indicate that the antiserum has a lower affinity for the protein from genome-negative Burkitt's lymphoma cells than for BNLF-1. It remains to be determined whether this cross-reacting protein from genome-negative cells is a cellular homolog of the viral protein or is merely a protein that binds to the antiserum by chance.

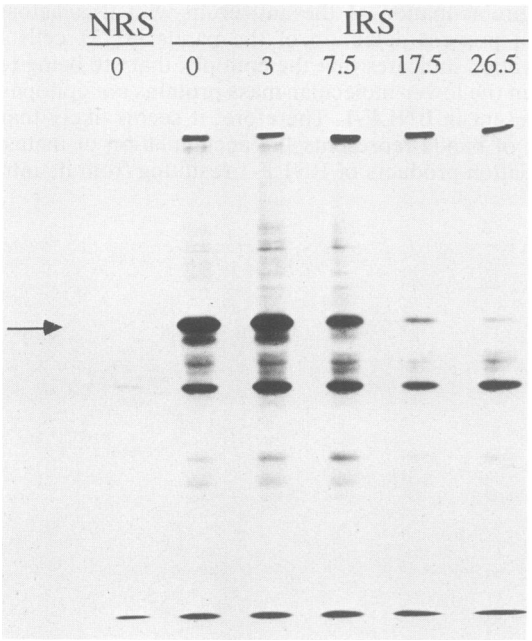

FIG. 6. Pulse-chase analysis of 721 cells labeled with [^{35}S]Met. 721 cells were labeled with [^{35}S]Met for 4 h. Previous experiments had indicated that incorporation of [^{35}S]Met into BNLF-1 was linear up to 5 h (data not shown). At the end of the labeling period, the cells were collected by centrifugation, transferred to fresh medium, and incubated at 37°C. At 0, 3, 7.5, 17.5, and 26.5 h after the start of the chase, cell samples were collected by centrifugation. Cell lysate from the 0-h time point was halved and immunoprecipitated with normal rabbit serum (NRS) or immunoaffinity-purified antiserum (IRS). For all other time points, lysates were immunoprecipitated with immunoaffinity-purified antiserum (IRS) only. Each polyacrylamide gel lane represents the equivalent of 5×10^6 cells labeled with 250 μCi of [^{35}S]Met at the start of the experiment. The arrow indicates the BNLF-1 protein. The other bands on the gel, in lanes marked IRS, represent nonspecific precipitation of proteins. Bands with similar mobilities are visible in the control lane (NRS) on longer fluorographic exposures of the gel. Details of the experiment are given in Results and Materials and Methods.

It has been possible to observe two posttranslational modifications of the BNLF-1 protein, phosphorylation and metabolic turnover, since the antiserum can immunoprecipitate BNLF-1 from cell lysates. Both BNLF-1 (Fig. 4) and the approximately 43K lytic protein are phosphorylated (data not shown). Phosphoserine is the only phosphoamino acid that is found to be associated with BNLF-1. However, if other phosphoamino acids were present at 1/10 the level of phosphoserine, they would not have been detected. A number of retroviral oncogene products are phosphoproteins and have an associated kinase activity (4); hence, we tested immunoprecipitates of BNLF-1 for an associated kinase activity. However, with the assay conditions that we used, we found no kinase activity associated with BNLF-1 (data not shown). For a number of phosphoproteins, the extent of phosphorylation is known to affect the activity of the protein (17). Therefore, identifying conditions that affect the extent of BNLF-1 phosphorylation might help in identifying conditions that modulate BNLF-1 activity.

BNLF-1 is turned over in 721 cells with a half-life of approximately 5 h (Fig. 6). In protein immunoblots, we see a ladder of bands extending up to approximately 62K in EBV genome-positive cell lines (Fig. 7). The lower-molecular-mass bands in this ladder most likely represent in vivo degradation products of BNLF-1. The ladder seen in the genome-positive cell lines 721 and 3/15-31 matches up with the ladder seen in COS cells expressing BNLF-1, and in the COS cells expressing BNLF-1 a ladder of bands is seen even in immunoprecipitates from [^{35}S]MET-labeled lysates (Fig. 2). Pulse-chase experiments indicated that in COS cells the 62K protein is turned over with a half-life of approximately 2 h and that some of the lower-molecular-mass proteins appear to be derived from the 62K protein (data not shown).

Why the ladder of bands seen in EBV genome-positive cells was not detected in earlier studies is not clear (11, 22). It is possible that in the different cell lines used in the different studies the protein is degraded to various extents. Alternatively, the antibodies used for immunoblots in the earlier studies might not have had sufficient affinity for the protein or might not have recognized sufficient epitopes on the protein to permit detection of the presumed degradation products. Yet another possibility is that the conditions that we have used to make cell lysates for protein immunoblots result in proteolysis of BNLF-1 in vitro. Although this explanation remains a formal possibility, as discussed

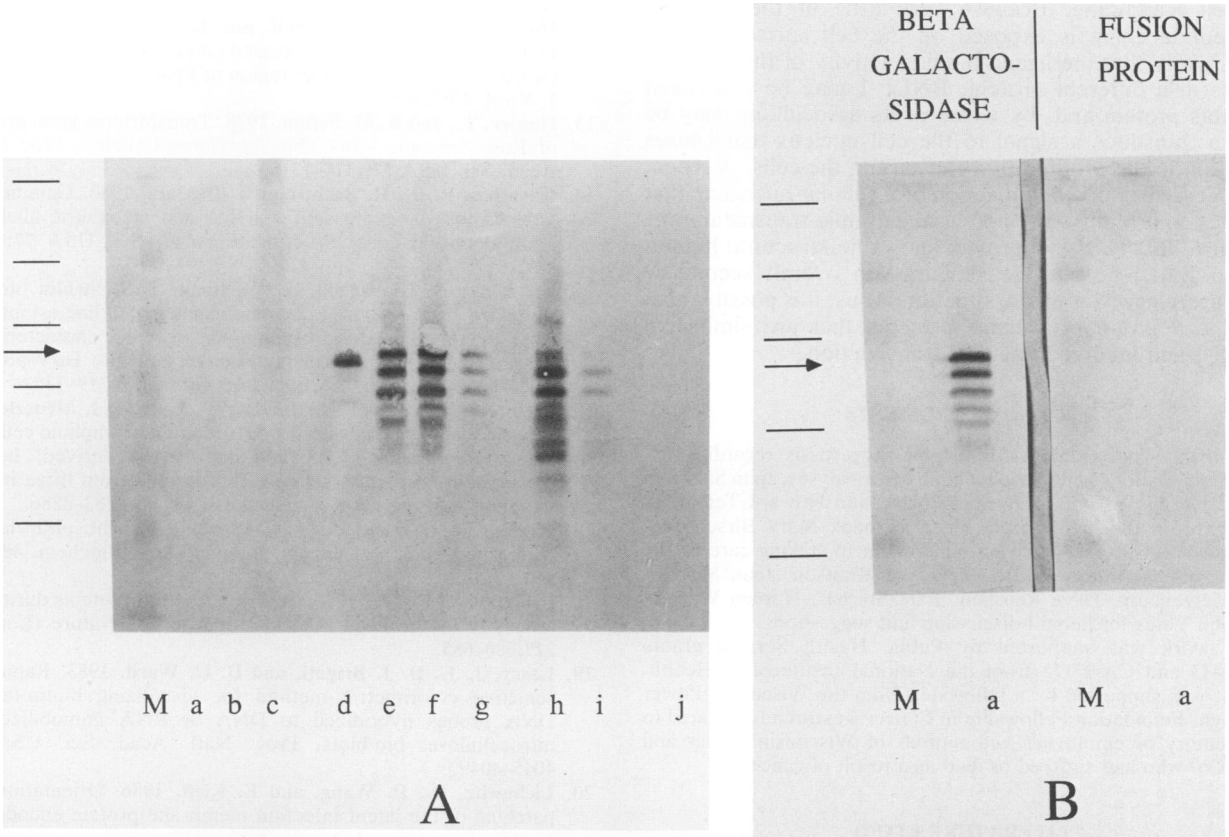

FIG. 7. Protein immunoblots with the immunoaffinity-purified antiserum. Cell lysates were resolved on a 10% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose and probed with a 1:100 dilution of the purified antiserum. Alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin was used as a detecting antibody at a dilution of 1:625. Color development was as described in Materials and Methods. The lines to the left and the arrows in panels A and B have the same meaning as in Fig. 2. (A) The gel lanes are molecular-mass markers (M) and cell lysates from 10^6 Manca cells (a), 10^6 Wilson cells (b), 10^6 BJAB cells (c), 10^5 Raji cells (d), 10^5 3/15-31 cells (e), 10^5 721 cells (f), 10^4 721 cells (g), 10^4 COS cells electroporated with $pSV_2BNLF-1$ (h), 10^3 COS cells electroporated with $pSV_2BNLF-1$ (i), and 10^4 COS cells electroporated with p264.2 plus pSV_2Neo (j). (B) Preincubation of immunoaffinity-purified immune serum with the fusion protein, but not β-galactosidase, prevents detection of the proteins from 721 cell lysates. Cell lysates from 10^5 721 cells were resolved on a gel lane (a), along with molecular-mass markers (M) in duplicate. After transfer to nitrocellulose, the nitrocellulose paper was cut in two. One piece was probed with immune serum that had been preincubated at 4°C for 45 min with 60 μg of β-galactosidase, and the other was probed with immune serum that had been preincubated with 60 μg of fusion protein.

above, we think it is unlikely to be the only explanation for the observations presented in Fig. 7.

The significance of the observations that indicate that BNLF-1 is turned over in virally transformed cells is not apparent. The half-life that is observed for BNLF-1 in 721 cells (5 h) is considerably shorter than the cell generation time of 20 to 24 h. The turnover rate of BNLF-1 is also considerably shorter than the average turnover rate for total cellular protein, which in these experiments is greater than 15 h. Thus, newly synthesized BNLF-1 is turned over within a cell cycle, and its half-life is shorter than the average half-life for total cellular protein. It should be noted that even though we interpret the loss of BNLF-1 signal in the pulse-chase analysis as metabolic turnover, we cannot rule out the possibility that some of this loss is due to conversion of newly synthesized BNLF-1 to a form that is lost in the preclearing pellet during immunoprecipitation. However, such a conversion cannot explain by itself the observed half-life of approximately 5 h, since no greater than 20 to 25% of the BNLF-1 present in the cell is lost during preclearing (data not shown; see Materials and Methods for details).

There are several possible explanations for this rapid turnover of BNLF-1 in virally transformed cells. One possibility is that BNLF-1 is required only at a particular period in the cell cycle and hence is synthesized during one phase of the cell cycle and then degraded. Another possibility is that the 62K BNLF-1 protein is a polyprotein; the lower-molecular-mass proteins may be derived from it and might represent distinct functional entities, as for example with the structural proteins of retroviruses (5). A third possibility is that turnover of the protein is associated with its activity. BNLF-1 is a membrane protein; growth factor receptors are membrane proteins that are endocytosed and degraded. For example, growth factor receptors, such as the IL-2 receptor (27) or the epidermal growth factor receptor (28), are internalized and presumably degraded. Thus, the turnover of BNLF-1 might result from endocytosis and lysosomal degradation. In this connection, it is interesting that immunofluorescence analysis of methanol-fixed EBV genome-positive cells indicate that BNLF-1, like growth factor receptors, is present in patches and caps in the membrane (20; unpublished observations). We therefore speculate that BNLF-1 may be part of a growth factor receptor complex in

the cell membrane. Because very little of the BNLF-1 polypeptide chain is exposed on the cell surface (20), it seems likely that the ligand-binding activity of the receptor resides in a different protein. BNLF-1 may be associated with this protein and, by virtue of its association, may be able to transduce a signal to the cell nucleus that causes alteration in the proliferative capacity of the cells. A recent study describes the identification of a cellular oncogene that encodes a protein with potential multiple-transmembrane domains (36). This oncoprotein shows no structural homology to BNLF-1, but the similarity in overall secondary structure suggests a similar function. Thus, it is possible that there is a group of cellular proteins that are similar to BNLF-1 and involved in signal transduction.

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