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LMP-1 Activates NF-κB by Targeting the Inhibitory Molecule IκBα

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LMP-1, an Epstein-Barr virus membrane protein expressed during latent infection, has oncogenic properties, as judged from its ability to transform B lymphocytes and rodent fibroblasts. LMP-1 induces the expression of *bcl2*, an oncogene which protects cells from apoptosis, as well as of genes encoding other proteins involved in cell regulation and growth control. The mechanisms by which LMP-1 upregulates these proteins is unknown, but it is plausible that LMP-1 modifies signal transduction pathways that result in the activation of one or more transcription factors that ultimately regulate transcription of oncogenic genes. NF-κB, a transcription factor controlling the expression of genes involved in cell activation and growth control, has been shown to be activated by LMP-1. The mechanism(s) regulating this activation remains unknown. Our data indicate that increased NF-κB DNA binding and functional activity are present in B-lymphoid cells stably or transiently expressing LMP-1. IκBα is selectively modified in LMP-1-expressing B cells. A phosphorylated form of IκBα and increased protein turnover-degradation correlate with increased NF-κB nuclear translocation. This results in increased transcription of NF-κB-dependent-genes, including those encoding p105 and IκBα (MAD3). These results indicate that LMP-1 activates NF-κB in B-cell lines by targeting IκBα. Identification of the pathways activated by LMP-1 to result in posttranslational modifications of IκBα will aid in determining the role of this virus-host cell protein interaction in Epstein-Barr virus-mediated oncogenesis.

Epstein-Barr virus (EBV) is a major cause of infectious mononucleosis and is a prototypic oncogenic virus. EBV has also been associated with Burkitt's lymphoma and a variety of B-cell lymphomas in immunocompromised individuals, notably posttransplant lymphoproliferative disease (17, 30, 43). Similar to other members of the herpesvirus family, EBV can remain in a latent state in B lymphocytes. During latent infection, different EBV proteins, including six nuclear proteins (EBNAs 1, 2, 3A, 3B, 3C, and LP), three integral membrane proteins (LMP-1, -2A, and -2B), and two small RNAs (EBER1 and -2), can be expressed (36). LMP-1 is a plasma membrane protein containing a transmembrane and a cytoplasmic domain which forms a tight patch within the cytoplasmic membrane (25, 26). Its individual role in promoting cell transformation has been clearly demonstrated: transfection of the LMP-1 gene is sufficient to transform rodent fibroblasts and normal human B lymphocytes, and EBV lacking the LMP-1 gene does not transform B lymphocytes (19, 41). The mechanisms mediating LMP-1-induced oncogenesis are yet to be elucidated. Evidence of cell activation (increase in CD23 and CD40 receptors) and upregulation of adhesion molecules (LFA1, LFA3, and ICAM1) is present in LMP-1-transfected cells, reflecting modulation of potential oncogenic mechanisms (42). Also, although the issue is controversial, LMP-1 has been directly associated with the activation of proto-oncogenes such as *bcl2* that render B lymphocytes resistant to apoptotic death (10, 14, 28, 34). Whether any of these mechanisms mediate LMP-1-induced oncogenesis is under study. Other viral proteins that convey transformation phenotypes, such as bovine papillomavirus E5, are similar in structure and membrane localization to LMP-1 and have been shown to interact with cytoplasmic membrane-associated growth factor receptors (32). LMP-1 interacts with vimentin, but it is not known if it interacts with other membrane-associated molecules that potentially regulate oncogenesis (24). It is therefore plausible that LMP-1 results in the modification of signal transduction pathways that ultimately regulate specific transcription factors involved in gene regulation and oncogenesis.

In this study, we have focused on NF-κB, a transcription factor shown to regulate a large number of genes involved in cell activation and growth control and to be activated by different viral proteins with oncogenic potential such as hepatitis B virus HBX protein and human T-cell leukemia virus type I (HTLV-I) Tax (16, 27). NF-κB belongs to a highly conserved family of transcription factors with N-terminal homology to the v-*rel* oncogene (implicated in avian lymphoblastoid tumor production) (23) that includes c-*rel*, p50 (NF-κB1), p52 (NF-κB2), p65 (*relA*), *relB*, and *dorsal* (reviewed in references 3 and 31). Each of these polypeptides can form homodimers or dimerize with other Rel family members, although the prototype NF-κB is a heterodimer composed of p50 and p65. The function of NF-κB is regulated by a series of inhibitory molecules named IκBs. The family of IκB molecules includes IκBα, IκBβ, IκBγ, p105, and p100 (reviewed in reference 31). The latter two molecules are also the precursors of p50 and p52, respectively (20, 35). IκB molecules function by sequestering NF-κB in the cytoplasm, rendering it inactive (2, 31, 44). Among the different IκBs, IκBα plays a major role in the regulation of NF-κB in lymphoid cells. Posttranslational modifications of IκBα induced by different stimuli and/or viral infection that activate different signal transduction pathways include its phosphorylation and subsequent proteolytic degradation. This causes the release of NF-κB, which translocates to the nucleus (1, 3, 4, 6, 7, 12, 15, 29, 31, 33, 37). Because the IκBα (MAD3) gene is NF-κB dependent (22), translocation of NF-κB secondary to IκBα proteolysis will upregulate the transcription of the IκBα gene to resynthesize and replenish the cytosolic IκBα pool, thus interrupting the cycle of NF-κB activation by anchoring p65 and p50 in the cytosol (22, 37).

In this study, we have analyzed whether and through which

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mechanisms NF-κB is regulated by the oncogenic EBV protein LMP-1 in B lymphocytes. To do so, we have used a variety of B-cell lines which stably express LMP-1, as well as models of transient LMP-1 expression. Our results indicate that LMP-1 activates NF-κB in B lymphocytes by targeting the phosphorylation and proteolysis of IκBα.

MATERIALS AND METHODS

Cell lines. Jurkat T-cell lines were purchased from the American Type Culture Collection B-cell line Ly1 was kindly provided by D. Jelinek, Mayo Clinic. The parental cell line BJAB is an EBV-negative African Burkitt's lymphoma-derived cell line. BjB3LT is a BJAB cell line stably expressing the LMP-1 gene; both cell lines were kindly provided by Georg Klein (Karolinska Institute, Stockholm, Sweden) (8). BJgp2 is a BJAB cell line stably expressing plasmid MTLM gpt-LMP-1, while its control cell line BJMTLM is the BJAB cell line stably transfected with plasmid MTLM gpt. BL41 is a Burkitt's lymphoma EBV-negative cell line, and BLgpt1 cells are BL41 cells stably expressing LMP-1. The two latter sets of cell lines were provided by Fred Wang (Harvard University). All of the cell lines specified above were found to be negative for *Mycoplasma* infection upon repetitive testing. Cells were grown in RPMI 1640 supplemented with 10% fetal calf serum, 200 μg of streptomycin per ml, and 200 U of penicillin per ml and passaged at an initial concentration of 0.1×10^6 cells per ml every 3.5 days. BJMTLM, BJgpt2, BL41, and BL41-gpt1 cells were passaged in the above-described culture medium supplemented with 3 μg of mycophenolic acid (Gibco-BRL, Gaithersburg, Md.) and xanthine-hypoxanthine (Sigma) per ml. For experiments, cells were passaged at 0.2×10^6 to 0.3×10^6 cells per ml and used 48 h later. BjB3LT cells were found to be refractory to serum deprivation-induced apoptosis. LMP-1 expression was determined by immunofluorescence and immunoprecipitation using CS1-4 antibodies (Dako, Copenhagen, Denmark).

Plasmids and plasmid construction. The κB-conalbumin-luciferase (κB-c-luc) plasmid contains three tandem copies of the κB motif of the human immunodeficiency virus (HIV) long terminal repeats cloned upstream of the minimal conalbumin promoter and luciferase gene (40). The thymidine kinase (TK)–β-galactosidase β-Gal plasmid is a reporter vector designed for expression of β-Gal in mammalian cells driven by the herpes simplex virus TK minimal promoter (Clontech, Palo Alto, Calif.). The LMP-1 gene inserted into the *Bam*HI site of pUC19 was kindly provided by Fred Wang and Elliott Kieff, Brigham and Women's Hospital, Boston, Mass. (42) and cloned by using the *Bam*HI site into pcDNA3 (Invitrogen, San Diego, Calif.), yielding pcDNA3-LMP-1. An antisense LMP-1 cloned in the same vector (pcDNA-1PML) was used as a control. MAD3 luc, MAD3 ΔκB-luc (22), and $p105^{luc}$ (40) were kindly provided by A. Israël, Paris, France. Plasmids were amplified in *Escherichia coli* DH5α and purified by using commercial kits (Qiagen, Chatsworth, Calif.).

Electroporation. Cell were electroporated in disposable sterilized cuvettes (PG220D; Hoefer, San Francisco, Calif.), using the Progenetor II electroporator (Hoefer Scientific Instruments) with the following settings: 300 V, 980 μF, and 1 s of discharge duration. Cells (10^7) were incubated during 10 min with 3 μg of κB-Luc or 5 μg of AP1-Luc and 5 μg of TK–β-Gal as reporter plasmids and variable amounts (as indicated in Results) of pcDNA3-LMP-1 or pcDNA3-1PML in 0.8 ml of serum-free RPMI medium. Following electroporation, cells were transferred to 10 ml of RPMI–10% fetal bovine serum medium and harvested between 16 and 24 h after electroporation, using 125 μl of cell culture lysis reagent (Promega, Madison, Wis.). Luciferase levels were measured in 100 μl by using the Promega luciferase assay system, and β-Gal activity was measured in 15 μl by using the Tropix Galacto-Light reporter assay (Tropix, Bedford, Mass.). Both luciferase and β-Gal were measured in a Berthold luminometer. Total protein in an aliquot of each sample was measured by the Bradford technique (Bio-Rad, Hercules, Calif.). Results are represented as luciferase units per β-Gal unit and are representative of at least four different sets of experiments.

Nuclear extracts and EMSA. Cytosolic and nuclear fractions were extracted by the method of Dignam et al. (9). Briefly, 5×10^6 to 20×10^6 cells were washed with phosphate-buffered saline and buffer A (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol [DTT], 2 μg of aprotinin per ml, 2 μg of leupeptin per ml, 2 μg of pepstatin per ml, 20 μg of Pefabloc per ml) to which 0.1% Nonidet P-40 was added for 10 min at 4°C. The pellet was resuspended in 20 to 30 μl of buffer C (20 mM HEPES, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, aprotinin, leupeptin, pepstatin, Pefabloc), incubated at 4°C for 30 min, and centrifugated. Fifty microliters of buffer D (20 mM HEPES, 20% [vol/vol] glycerol, 0.05 M KCl, 0.2 mM EDTA, 0.5 mM DTT, aprotinin, leupeptin, pepstatin, Pefabloc) was added to the supernatant. Electrophoretic mobility shift assays (EMSAs) were performed with 3 to 4 μg of nuclear protein, incubating the protein with 4 μg of poly(dI-dC) and 1 μg of sonicated single-stranded DNA (Pharmacia, Piscataway, N.J.) in a total volume of 15 to 20 μl of binding buffer for 15 min. Then the mixture was incubated for 5 min with antibody or nonlabeled oligonucleotide and with 70,000 to 100,000 cpm of [γ-^{32}P]ATP-labeled double-stranded oligodeoxynucleotide probe at room temperature for 15 min. The product of this reaction was analyzed by electrophoresis in a nondenaturing 5% polyacrylamide gel and visualized by autoradiography. DNA binding competition was assessed by preincubating the nuclear extract with a 40-fold excess of unlabeled oligonucleotide for 5 min. One microliter of anti-NF-κB antibodies (anti-p65, anti-p50, anti-p52, and anti-c-Rel) (Santa Cruz Biotechnology, Santa Cruz, Calif.) was added to supershift DNA-protein complexes. Oligonucleotides used in EMSA corresponded to the NF-κB binding sequence present within the enhancer of the HIV long terminal repeat (5′-ACAAGGGACTTTCCGCTGG GGACTTTCCAGGGA-3′), to the consensus sequence of AP1 (5′-CGCTTG ATGAGTCAGCCGGAA-3′), and CREB (5′-AGAGATTGCCTGACGTCAG AGAGCTAG-3′).

Western blots (immunoblots). Cytosolic proteins were extracted in the same procedure as nuclear extracts after lysing the cells with buffer A with 0.1% Nonidet P-40. The resultant supernatant contained cytosolic proteins. Proteins (cytosolic and nuclear) were separated by electrophoresis on sodium dodecyl sulfate (SDS)–10 to 12.5% polyacrylamide gels and transferred to an Immobilon-P membrane (Millipore, Bedford, Mass.) Immunoblotting was performed with commercially available anti-p65, p50, p100, and c-Rel antibodies (Santa Cruz Biotechnology) and visualized by using an ECL (enhanced chemiluminescence) Western blotting detection kit (Amersham, Arlington Heights, Ill.).

RNA extraction and Northern (RNA) blot analysis. Ten micrograms of total cellular RNA was electrophoresed in an agarose-formamide 0.9% gel (2 h, 60 V), transferred overnight to a Hybond-N+ membrane (Amersham) by osmosis, and hybridized with ^{32}P-labeled probes. The IκBα (MAD3) probe consisted of a 1.3-kb *Eco*RI-*Xho*I fragment of the *MAD3* cDNA, the p105 probe is a 1.9-kb *Pst*I fragment, and the *GAPDH* probe is a 1.3-kb *Pst*I fragment. Probes were labeled with [α-^{32}P]dCTP by using a random primer DNA labeling kit (Boehringer Mannheim, Indianapolis, Ind.).

[^{35}S]methionine-cysteine labeling, chase, and immunoprecipitation. Cells (1.5×10^7 per point) at a concentration of 2×10^6 cells per ml were incubated in preconditioned methionine- and cysteine-free medium (ICN, Irvine, Calif.) for 4 h in T25 flasks and then labeled with 0.5 mCi of ^{35}S-labeled methionine and cysteine (Translabel; ICN) per 10^7 cells for 2 h at 37°C. The culture supernatant was aspirated, and 10 ml of regular medium supplemented with L-methionine and L-cysteine (10 mM) was added. Cells were incubated in this medium for different lengths of time and harvested by using lysis buffer (50 mM Tris, 50 mM NaCl, 1% Triton X-100, 2 mM EDTA, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mg of *p*-nitrophenyl phosphate per ml, 30 mM pyrophosphate, 10 μg of β-glycerolphosphate per ml, 2 μg of aprotinin per ml, 2 μg of leupeptin per ml, 2 μg of pepstatin per ml [pH 7.5]). After centrifugation, the supernatant was incubated overnight at 4°C with anti-IκBα antibody (Santa Cruz Biotechnology). Protein G-agarose beads (Gibco-BRL) were added to each sample, the samples were incubated for 2 h at 4°C and washed, and the protein was eluted as previously described. These eluants were separated by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to an Immobilon-P membrane (Millipore) as described above for Western blots, and visualized by autoradiography.

For calf intestinal phosphatase (CIP) treatment, 1.5×10^7 cells were [^{35}S]methionine-cysteine labeled, lysed, and immunoprecipitated as described above. Immunoprecipitates were treated with 33 U of CIP (Sigma, St. Louis, Mo.) or mock treated in 50 μl of 50 mM Tris HCl (pH 8)–1 mM $MgCl_2$ at 32°C for 15 min, analyzed by SDS-PAGE, and visualized by autoradiography.

RESULTS

Transient expression of LMP-1 increases NF-κB activity. We first tested whether transient LMP-1 expression in the EBV-negative cell lines BJAB, Ly1, and Jurkat resulted in NF-κB activation. pcDNA-LMP-1 or its antisense control were separately cotransfected with the κB-c-luc construct and a TK–β-Gal reporter to normalize transfection efficiency. As shown in Fig. 1A, LMP-1 increases the luciferase expression driven by the κB concatemer, while the control plasmid does not affect the basal NF-κB–luciferase activity. Neither LMP-1 or its antisense control plasmid modified the basal activity of the AP1-c-luc construct or of the minimal conalbumin promoter-luciferase gene. To confirm that LMP-1 increases NF-κB activation, we next determined whether transient expression of this viral protein increases the DNA binding activity of NF-κB. For this, LMP-1 or its antisense control plasmid was electroporated into BJAB cells. Eighteen hours later, cells were harvested and the cytosolic and nuclear fractions were isolated. As shown in Fig. 1B, transfection of an antisense LMP-1 plasmid does not modify NF-κB DNA binding (lane 1), while increased NF-κB DNA binding is present in LMP-1 gene-transfected cells (lane 2). This protein complex binding DNA is specifically competed for with an excess of unlabeled NF-κB oligonucleotide (lane 3). No increased DNA binding was observed in

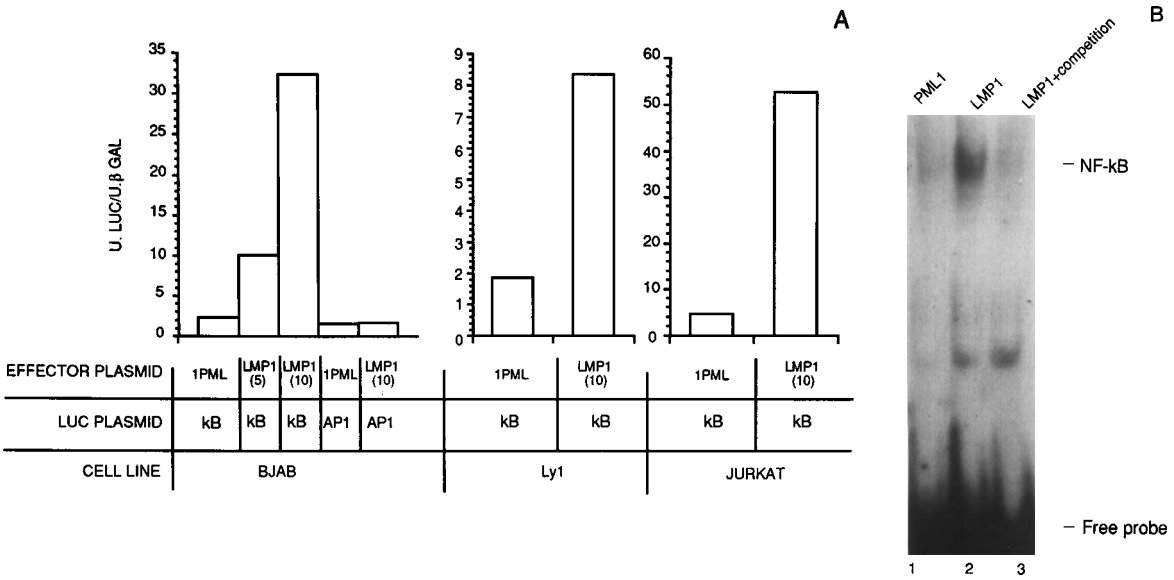

FIG. 1. Transient expression of LMP-1 increases NF-κB activity. (A) Cotransfection of 10^7 BJAB, Ly1, and Jurkat cells with pcDNA3-1PML (10 μg) and 5 or 10 μg of pcDNA3-LMP-1 and a κB-c-luc or AP1-c-luc reporter gene. Results are expressed in luciferase (LUC) light units normalized to β-Gal units and are representative of three experiments. (B) BJAB cells (10^7) were transfected with 10 μg of pcDNA3-1PML (lane 1) or pcDNA3 LMP-1 (lane 2), nuclear protein was extracted, and EMSA was performed. NF-κB and free probe are indicated at the right.

nuclear fractions from LMP-1 or control transfected cells when a labeled AP1 oligonucleotide was used (data not shown). Altogether, these results suggest that transient expression of the EBV LMP-1 protein results in NF-κB activation.

NF-κB activation is increased in B-cell lines stably expressing LMP-1. To further study in detail the mechanisms by which LMP-1 activates NF-κB in B lymphocytes, we used three B-cell lines that stably express this viral protein: BjB3LT, BJgpt2, and BLgpt1.

We first tested whether NF-κB activity was greater in nuclear extracts from the LMP-1-positive (BjB3LT, BJgpt2, and BLgpt1) than in nuclear extracts from the parental LMP-1-negative cell lines (BJAB, BJMTLM, and BL41, respectively). As shown in Fig. 2A, NF-κB DNA binding was greater in the nuclei of LMP-1-positive cells than in their respective LMP-1-negative controls. The basal NF-κB DNA binding activity present in BJAB cells increases following cell stimulation with phorbol myristate acetate and lipopolysaccharide (LPS) (data not shown). To confirm that the increased NF-κB DNA binding activity in BjB3LT cells is specific to NF-κB, binding to AP1 and CREB motifs was tested in the same nuclear extracts of BJAB and BjB3LT cells by EMSA using the corresponding labeled oligonucleotides. As shown in Fig. 2A, no significant increase in AP1 DNA binding activity was observed, but a mild increase in the DNA binding to the CREB motif was found. Each protein-DNA complex was competed for with an excess of the corresponding unlabeled oligonucleotide. Altogether, these results confirm those obtained by transiently expressing LMP-1 in B cells, indicating that LMP-1 activates NF-κB.

To determine whether the increased NF-κB DNA binding activity present in BjB3LT cells correlates with increased NF-κB-dependent transcriptional function, the κB-c-luc reporter gene was cotransfected with a TK–β-Gal reporter gene into LMP-1-positive and LMP-1-negative cell lines. Once more, greater NF-κB activity was observed in the LMP-1-positive B cells (BjB3LT, BLgpt1, and BJgpt2) than in LMP-1-negative parental cells (BJAB, BL41, and BJMTLM, respectively) (Fig. 2B). The basal transcriptional activities of the minimal conal-

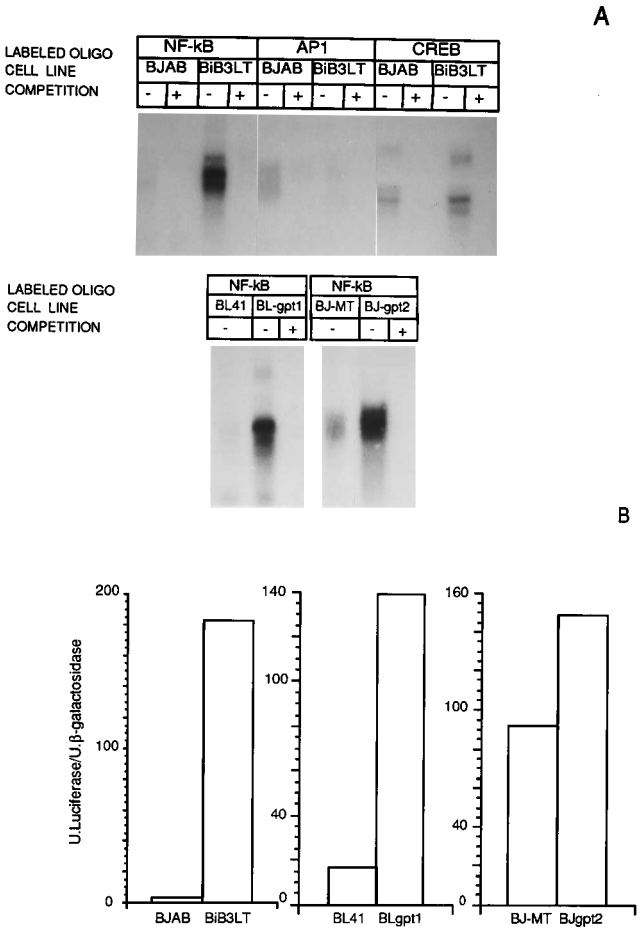

FIG. 2. NF-κB activation is increased in B-cell lines stably expressing LMP-1. (A) EMSA using labeled NF-κB, AP1, and CREB oligonucleotides with nuclear extracts from BJAB and BjB3LT cells or labeled NF-κB oligonucleotides with nuclear extracts from BL41, BLgpt1, BJMTLM, and BJgpt2 cells. (B) Transfection of 10^7 BJAB, BjB3LT cells, BL41, BLgpt1, BJMTLM, and BJgpt2 cells with κB-c-luc.

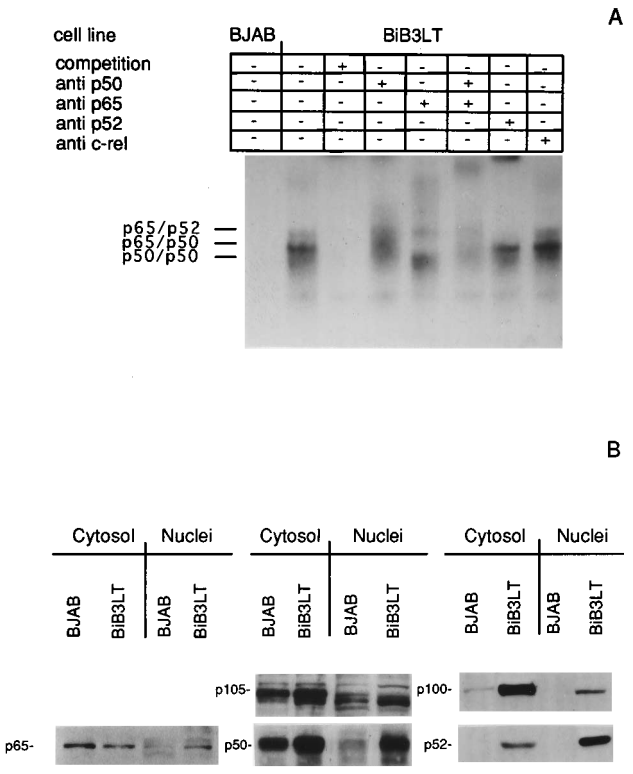

FIG. 3. (A) EMSA of nuclear extracts from BJAB and BjB3LT cells, using a ^{32}P-labeled NF-κB oligonucleotide. Nuclear proteins were incubated with unlabeled nucleotide or antibodies against p65, p50, p52, or c-Rel. The positions of p65-p50, p65-p52 and p50-p50 complexes are indicated at the left. (B) Western blot from cytosolic and nuclear extracts of BJAB and BjB3LT cells followed by immunoblotting. Signals corresponding to p65, p105, p50, p100, and p52 are marked at the left.

bumin promoter-luciferase and AP1-conalbumin-luciferase reporter genes were similar in the two types of cell lines (data not shown).

The molecular forms of NF-κB activated by LMP-1 include p65, p50, and p52. In different combinations among proteins of the Rel family, a variety of homo- and heterodimers are formed, each of which is regulated by specific IκB molecules (reviewed in references 3 and 31). To study the mechanisms regulating LMP-1-mediated NF-κB activation, we first determined the composition of the NF-κB-like complex present in the nuclei of BjB3LT cells. Nuclear extracts of these cells and of control BJAB cells were analyzed in EMSA using specific commercial antibodies to the different Rel protein members. Using a labeled oligonucleotide encompassing the NF-κB binding motif, we demonstrate that the DNA-binding complexes include p65/p50 and p52 but not c-Rel. Antibodies to p65, p50, and p52 resulted in supershifts of the DNA-binding protein complex (Fig. 3A). This was not observed when anti-c-Rel antibodies were used.

We next confirmed the composition of the NF-κB nuclear complex by analyzing the steady-state protein levels of the different NF-κB subunits in both cytosolic and nuclear fractions, using Western immunoblotting techniques (Fig. 3B). p65 protein levels were present in the nuclear fraction of BjB3LT cells but not in that of the control BJAB cells, suggesting continuous p65 nuclear translocation in BjB3LT cells. Steady-state levels of the precursor p105 and its proteolytic product p50 are higher in the cytosolic fraction of BjB3LT cells than in that of BJAB cells. Moreover, p50 levels are also higher in the nuclear fraction of BjB3LT cells than in that of BJAB cells. A protein migrating faster than p105 is faintly detected in nuclear extracts of both cell types, using anti-p105 antibodies. The origin of this protein is unknown, although we cannot exclude some degree of cytosolic contamination. Results to those similar for p105 and p50 were obtained when p100 and p52 were analyzed in BjB3LT cells (Fig. 3B).

Altogether, EMSA and immunoblotting experiments indicate that LMP-1-positive cells contain increased NF-κB nuclear translocation probably secondary to the regulation of IκB molecules that target p65/p50 and p65/p52 heterodimers such as IκBα and/or p105/p100. Increased steady-state levels of p105 and p100 in BjB3LT cells suggest their increased synthesis by NF-κB-dependent mechanisms, since both molecules are regulated by NF-κB at the transcriptional level.

IκBα phosphorylation and proteolysis are increased in LMP-1-expressing cells. IκBα sequesters NF-κB by masking the nuclear localization signal of p65 (5, 11). Posttranslational modifications of IκBα such as phosphorylation and/or proteolysis result in NF-κB and IκBα dissociation (4, 6, 7). To determine whether IκBα is selectively modified in BjB3LT cells, thus accounting for the continuous NF-κB translocation induced by LMP-1, we addressed its phosphorylation state and proteolytic processing. In vitro, IκBα phosphorylation is necessary for NF-κB/IκBα dissociation (12). In vivo, IκBα phosphorylation is detected prior to its proteolytic degradation following cell activation by "punctual" stimuli (e.g., LPS or tumor necrosis factor) (4, 6, 7). However, it is unknown whether the continuous presence of LMP-1 in B cells results in persistent modifications of IκBα.

To study the phosphorylation characteristics of IκBα in LMP-1-positive and LMP-1-negative cells, we first performed immunoprecipitation experiments with anti-IκBα antibodies. IκBα immunoprecipitated from LMP-1-positive cells (BJgpt2, BLgpt1, and BjB3LT) contained an additional, slower-migrating form of IκBα than the parenteral LMP-1-negative cells (BJMTLM, BL41, and BJAB, respectively) (Fig. 4A). To determine whether this slower-migrating form of IκBα present in LMP-1-positive cells is phosphorylation dependent, BjB3LT and the control BJAB cell lines were labeled with [^{35}S]methionine-cysteine in the presence of okadaic acid, a phosphatase inhibitor shown to sustain the phosphorylated state of IκBα following punctual stimuli (39). Cells were then harvested, IκBα was immunoprecipitated in a lysis buffer containing phosphatase inhibitors and competitors, and the samples were treated with CIP. Results from these experiments (Fig. 4B) indicate that increased levels of ^{35}S-IκBα are present in BjB3LT cells (compare lane 3 with lane 1) and that a slower-migrating form of IκBα is immunoprecipitated with anti-IκBα antibodies in BjB3LT cells but not in BJAB cells (lane 3). This slower-migrating form reverts into the faster-migrating complex upon CIP treatment (lane 4). These results indicate that IκBα becomes phosphorylated in BjB3LT cells, which probably renders the molecule susceptible to subsequent proteolysis. The latter possibility is partially supported by the fact that increased levels of ^{35}S-IκBα but not of steady-state protein are observed in the same experiment (compare lanes 5 to 8 with lanes 1 to 4).

To confirm increased proteolysis of IκBα in LMP-1-expressing cells, a ^{35}S pulse-chase labeling experiment was performed. A 2-h pulse again demonstrates higher ^{35}S levels of IκBα in BjB3LT cells than in BJAB cells (Fig. 4C, lanes 1 and 4). Chase with an excess of unlabeled cysteine and methionine for 60 and 150 min indicates that ^{35}S-IκBα levels decrease at a faster rate in BjB3LT cells than in BJAB cells (compare lanes 5 and 6 with

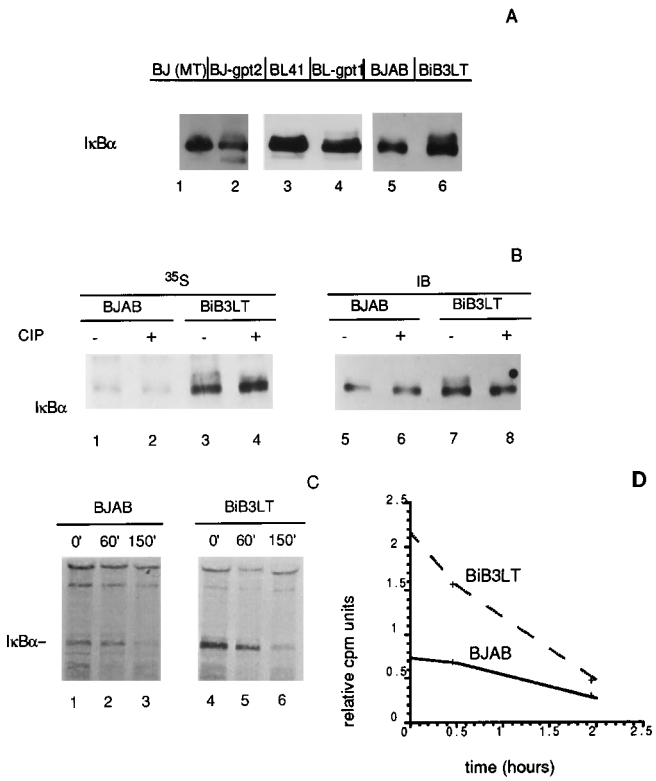

FIG. 4. IκBα is phosphorylated and its synthesis and proteolysis are increased in LMP-1-transfected cells. (A) Immunoprecipitates of IκBα from LMP-1-positive cells (BJgpt2, BLgpt-1, and BjB3LT) and their parental control cells (BJMTLM, BL41, and BJAB, respectively) were analyzed by SDS-PAGE, transferred to Immobilon membranes, and immunoblotted with anti-IκBα antibodies, using ECL (Amersham). (B) Immunoprecipitation (^{35}S) of IκBα from [^{35}S]Met-Cys-labeled BJAB and BjB3LT cells, separation by SDS-PAGE, transfer to a polyvinylidene difluoride membrane, and autoradiography. The polyvinylidene difluoride membrane was subsequently washed and immunoblotted (IB) with anti-IκBα antibodies and detected by ECL. (C) BJAB and BjB3LT [^{35}S]Met-Cys-labeled cells were pulsed for 2 h and harvested lanes 1 and 4) or chased for 60 (lanes 2 and 5) and 150 (lanes 3 and 6) min with an excess of unlabeled Met-Cys. This procedure was followed by cell lysis and immunoprecipitation of IκBα. This molecule is indicated at the left. (D) Densitometry of IκBα from a experiment similar to that described for panel B, using an AMBIS reader. IκBα and an unrelated protein were scanned, and the counts per minute of IκBα was normalized to the counts per minute of the irrelevant protein per lane. This experiment is representative of four different experiments.

lanes 2 and 3). The half-lives of IκBα were calculated to be 70 and 100 min for BjB3LT and BJAB cells, respectively (Fig. 4C). These results indicate that increased IκBα synthesis is followed by increased degradation (probably secondary to a qualitative phosphorylation modification) in LMP-1-positive cells. This results in similar steady-state levels of IκBα in the two cell lines.

Increased transcriptional activity of p105 and IκBα (MAD3) in LMP-1-expressing cells. p105 and IκBα are transcriptionally regulated by NF-κB (22, 40). This mechanism compensates for their usage during the activation cycle of NF-κB (37). To study whether the regulatory loop between NF-κB, p105, and IκBα is present in BjB3LT cells, Northern blot analysis of total cellular RNA from these and control cells (BJAB) was performed with p105 and IκBα (MAD3) probes. As shown in Fig. 5A, increased steady-state levels of both p105 and IκBα are present in BjB3LT cells. To confirm that the increased IκBα RNA level is secondary to an increased transcriptional activity of the enhancer-promoter gene, transfection of the wild-type MAD3 enhancer-promoter cloned upstream of the luciferase reporter gene (MAD3 luc) or a MAD3 enhancer-promoter luciferase gene lacking the NF-κB binding motif was performed. Luciferase activity of MAD3 luc is greater in BjB3LT cells than in parental BJAB cells. Deletion of the κB motif within the enhancer region significantly reduces the luciferase activity, suggesting that its increased transcriptional activity in BjB3LT cells is NF-κB dependent. p105 transcription regulation is also NF-κB dependent (40). To confirm that increased p105 protein levels and RNA present in LMP-1-positive cells was due to increased transcription of the p105 enhancer-promoter region, a luciferase construct containing this regulatory region was cotransfected with a TK–β-Gal reporter gene into BJAB and BjB3LT cells. As shown in Fig. 5B, the transcriptional activity of the promoter-enhancer region of p105 is also upregulated in the LMP-1-positive cells, BjB3LT. Because both p105 and the related protein p100 have IκB-like functions and when processed by proteolytic mechanisms yield p50 and p52, respectively, we determined whether this processing was increased in LMP-1-positive cells. In pulse-chase methionine-cysteine labeling experiments followed by immunoprecipitation of p105/p50 and p100/p52, we determined that the half-lives of both precursors and the proteolytic processing into p50 and p52 were the same in LMP-1-positive and control (LMP-1-negative) cells despite increased synthesis of both p105 and p100 in LMP-1-positive cells (data not shown).

Altogether, these data indicate that LMP-1 activates NF-κB at least by inducing posttranslational modifications of IκBα. This results in a regulatory loop that involves increased NF-κB translocation, which results in increased synthesis of p105, p100, and IκBα in an attempt to compensate for the continuous utilization of this relevant transcription factor and its inhibitory molecule.

DISCUSSION

Although the EBV LMP-1 protein is sufficient for cell transformation (19, 41), the exact mechanisms regulating virus-induced oncogenesis remain incompletely characterized. Viral proteins with oncogenic capabilities other than LMP-1 such as hepatitis B virus HBx protein and HTLV-I Tax also result in NF-κB activation (13, 16, 18, 21, 27, 34, 38). In this study, we have characterized the biochemical and molecular mechanisms by which LMP-1 activates NF-κB. These results will aide future investigations to establish the role of NF-κB in LMP-1-mediated oncogenesis and activation of lymphoid genes involved in cell activation and growth regulation.

Although a large body of information is now available as to how NF-κB and related proteins interact with their respective IκB molecules, the signal transduction pathways leading to NF-κB activation in B lymphocytes remain relatively unexplored. To identify the signal transduction pathways activated by LMP-1 to result in NF-κB activation, we first addressed which Rel/NF-κB molecules and IκBs were selectively modified by LMP-1. Identification of a specific IκB as responsible for LMP-1-mediated NF-κB activation would provide a readout for studying upstream pathways leading to modification of such IκBs. Our data indicate that LMP-1 results in the nuclear translocation of p65 in conjunction with p50 and p52. Because c-Rel was found to be present at equal levels in both LMP-1-negative and LMP-1-positive B-cell lines, it was inferred that this protein and its inhibitor molecule (IκBβ?) were not targets of LMP-1. Therefore, our studies focused on IκB molecules that regulate p65 such as IκBα and the precursor molecules p100 and p105. Results from such studies indicate that IκBα

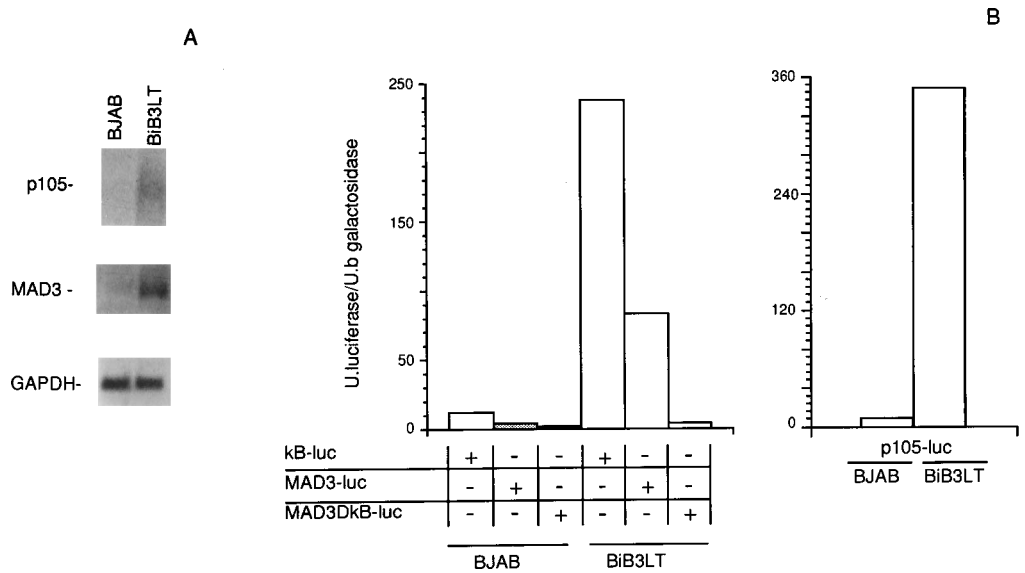

FIG. 5. Increased transcription of IκBα (MAD3) and p105 in BjB3LT cells. (A) Northern blot analysis of total cellular RNA (10 μg) from BJAB and BjB3LT cells. The probe is marked at the left. (B) Transfection of MAD3-c-luc, MAD3 ΔκB-c-luc, and p105 luc into 10^7 BJAB and BjB3LT cells. Results are expressed as luciferase units normalized to β-Gal units.

but not p100 or p105 was selectively modified by LMP-1. Although little information is available as to how a chronic stimulus, such as persistent expression of LMP-1, may modify IκBα to result in continuous NF-κB activation, our results suggest that LMP-1 causes continuous modifications of IκBα by at least two mechanisms: phosphorylation and proteolysis. These two events resemble IκBα modifications induced by punctual stimuli such as tumor necrosis factor or LPS. Similar data have recently been reported for a model of HTLV-I Tax (38). Although it is unknown whether IκBα phosphorylation in vivo is the triggering event resulting in its subsequent degradation, data generated from other cell models indicate that IκBα phosphorylation precedes and is required for proteolysis, even though the latter event ultimately causes NF-κB release (35a). Studies are currently under way to test this sequential mechanism in LMP-1-positive cells. Nevertheless, identification of IκBα phosphorylation mediated by LMP-1 as a key event in NF-κB regulation is a first step in identifying the signal transduction pathways triggered by LMP-1 and provides a first example of how a viral protein may result in continuous modification of IκBα. We hypothesize that phosphorylated IκBα becomes susceptible to degradation, which would explain the increased NF-κB translocation observed in our model. The B lymphocyte, in order to compensate for the shorter half-life of IκBα, uses the NF-κB-dependent regulatory loop to increase IκBα transcription and synthesis; this ultimately results in a balanced steady-state level of IκBα protein. Of interest, this situation is not observed in another model of chronic NF-κB activation such as that of persistent HIV infection in monocytes and macrophages: IκBα is selectively phosphorylated and degraded by HIV, but the monocytic cell is unable to completely compensate for IκBα utilization, thus resulting in decreased steady state levels of IκBα (28a). For the reasons cited above, LMP-1 expressing B-lymphocyte cell lines provide a useful model for the identification of the kinase/phosphate and protease(s) that regulate IκBα and NF-κB activation in B lymphocytes. Even though a large body of results was obtained for cell lines stably expressing LMP-1, many of the observations have also been confirmed by transient expression of LMP-1. This fact, together with recent published reports in which multiple cell lines were tested for the ability of LMP-1 to activate NF-κB (34), strengthens the conclusion that LMP-1 activates NF-κB and it is not due to an artifact derived from studying a specific cloned cell line in which increased NF-κB activation is a function not of LMP-1 but of intrinsic differences within such clone.

Although our results have identified IκBα as a target of LMP-1, other IκB molecules such as p105 and p100 that also regulate the NF-κB complex could have also been selectively modified in LMP-1-expressing cells. Our studies indicate that even though p105 and p100 are indirectly regulated by LMP-1 via NF-κB (increased steady-state protein levels secondary to increased gene transcription), they do not appear to be modified in a way that results in enhanced NF-κB translocation. The increased steady-state levels of both p105 and p100 in LMP-1-expressing B cells appears to be secondary to increased synthesis and to an increased rate proteolysis into p50 and p52, which are similar in the two cell lines.

Even though it is unclear what direct impact LMP-1-mediated NF-κB activation has in virus-induced oncogenesis, identification of IκBα as a target of LMP-1 provides a foundation for investigating pathways upstream of IκBα that may be activated by LMP-1. Because NF-κB activation appears to be an early event triggered by LMP-1, this model will potentially be useful for studying proximal second messenger-signal transduction pathways that can mediate LMP-1-induced oncogenesis.

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REFERENCES

1. **Baeuerle, P. A., and D. Baltimore.** 1988. Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-κB transcription factor. Cell **53:**211–217.
2. **Baeuerle, P. A., and D. Baltimore.** 1988. IκB: a specific inhibitor of the NF-κB transcription factor. Science **242:**540–546.
3. **Baeuerle, P. A., and T. Henkel.** 1994. Function and activation of NF-κB in the immune system. Annu. Rev. Immunol. **12:**141–179.
4. **Beg, A. A., T. S. Finco, P. V. Nantermet, and A. S. Baldwin, Jr.** 1993. Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of IκBα: a mechanism for NF-κB activation. Mol. Cell. Biol. **13:**3301–3310.
5. **Beg, A. A., S. M. Ruben, R. I. Scheinman, S. Haskill, C. A. Rosen, and A. S. Baldwin, Jr.** 1992. IκB interacts with the nuclear localization sequences of the subunits of NF-κB: a mechanism for cytoplasmic retention. Genes Dev. **6:**1899–1913.
6. **Brown, K., S. Park, T. Kanno, G. Franzoso, and U. Siebenlist.** 1993. Mutual regulation of the transcriptional activator NF-κB and its inhibitor, IκB-α. Proc. Natl. Acad. Sci. USA **90:**2532–2536.
7. **Cordle, S. R., R. Donald, M. A. Read, and J. Hawiger.** 1993. Lipopolysaccharide induces phosphorylation of MAD3 and activation of c-rel and related NF-κB proteins in human monocytic THP-1 cells. J. Biol. Chem. **268:**11803–11810.
8. **Cuomo, L., P. Trivedi, F. Wang, G. Winberg, G. Klein, and M. G. Masucci.** 1990. Expression of the Epstein-Barr virus (EBV)-encoded membrane antigen (LMP) increases the stimulatory capacity of EBV-negative B lymphoma lines in allogeneic mixed lymphocyte cultures. Eur. J. Immunol. **20:**2293.
9. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11:**1475–1489.
10. **Finke, J., R. Fritzen, P. Ternes, P. Trivedi, K. J. Bross, W. Lange, R. Mertelsmann, and G. Dolken.** 1992. Expression of bcl-2 in Burkitt's lymphoma cell lines: induction by latent Epstein-Barr virus genes. Blood **80:** 459–469.
11. **Ganchi, P. A., S.-C. Sun, W. C. Greene, and D. W. Ballard.** 1992. IκB/MAD-3 masks the nuclear localization signal of NF-κB p65 and requires the transactivation domain to inhibit NF-κB p65 DNA binding. Mol. Biol. Cell. **3:**1339–1352.
12. **Ghosh, S., and D. Baltimore.** 1990. Activation *in vitro* of NF-κB by phosphorylation of its inhibitor IκB. Nature (London) **344:**678–682.
13. **Hammarskjöld, M.-L., and M. C. Simurda.** 1992. Epstein-Barr virus latent membrane protein transactivates the human immunodeficiency virus type 1 long terminal repeat through induction of NF-κB activity. J. Virol. **340:**6496–6501.
14. **Henderson, S., M. Rowe, C. Gregory, D. Croom-Carter, F. Wang, R. Longnecker, E. Kieff, and A. Rickinson.** 1991. Induction of bcl-2 expression by Epstein-Barr virus latent membrane protein 1 protects infected B cells from programmed cell death. Cell **65:**1107–1115.
15. **Henkel, T., T. Machleidt, I. Alkalay, M. Krönke, Y. Ben-Neriah, and P. A. Baeuerle.** 1993. Rapid proteolysis of IκB-α is necessary for activation of transcription factor NF-κB. Nature (London) **365:**182–185.
16. **Hirai, H., T. Suzuki, J. Fujisawa, J. Inoue, and M. Yoshida.** 1994. Tax protein of human T-cell leukemia virus type I binds to the ankyrin motifs of inhibitory factor kappa B and induces nuclear translocation of transcription factor NF-κB proteins for transcriptional activation. Proc. Natl. Acad. Sci. USA **91:**3584–3588.
17. **Joske, D., and H. Knecht.** 1993. Epstein-Barr virus in lymphomas: a review. Blood Rev. **7:**215–222.
18. **Kanno, T., K. Brown, G. Franzoso, and U. Siebenlist.** 1994. Kinetic analysis of human T-cell leukemia virus type I *tax*-mediated activation of NF-κB. Mol. Cell. Biol. **14:**6443–6451.
19. **Kaye, K. M., K. M. Izumi, and E. Kieff.** 1993. Epstein-Barr virus latent membrane protein 1 is essential for B-lymphocyte growth transformation. Proc. Natl. Acad. Sci. USA **90:**9150–9154.
20. **Kieran, M., V. Blank, F. Logeat, J. Vandekerckhove, F. Lottspeich, O. Le Bail, M. B. Urban, P. Kourilsky, P. A. Baeuerle, and A. Israël.** 1990. The DNA binding subunit of NF-κB is identical to factor KBF1 and homologous to the rel oncogene product. Cell **62:**1007–1018.
21. **Laherty, C. D., H. M. Hu, A. W. Opipari, F. Wang, and V. M. Dixit.** 1992. The Epstein-Barr virus LMP1 gene product induces A20 zinc finger protein expression by activating nuclear factor kappa B. J. Biol. Chem. **267:**24157–24160.
22. **Le Bail, O., R. Schmidt-Ullrich, and A. Israel.** 1993. Promoter analysis of the gene encoding the IκBa(MAD3) inhibitor of NF-κB: positive regulation by members of the *rel*NF-κB family. EMBO J. **12:**5043–5049.
23. **Lewis, R. B., J. McClure, B. Rup, D. W. Niesel, R. F. Garry, J. D. Hoelzer, K. Nazerian, and H. R. Bose, Jr.** 1981. Avian reticuloendotheliosis virus: identification of the hematopoietic target cell for transformation. Cell **25:** 421–431.
24. **Liebowitz, D., R. Kopan, E. Fuchs, J. Sample, and E. Kieff.** 1987. An Epstein-Barr virus transforming protein associates with vimentin in lymphocytes. Mol. Cell. Biol. **7:**2299–2308.
25. **Liebowitz, D., J. Mannick, K. Takada, and E. Kieff.** 1992. Phenotypes of Epstein-Barr virus LMP1 deletion mutants indicate transmembrane and amino-terminal cytoplasmic domains necessary for effects in B-lymphoma cells. J. Virol. **66:**4612–4616.
26. **Liebowitz, D., D. Wang, and E. Kieff.** 1986. Orientation and patching of the latent infection membrane protein encoded by Epstein-Barr virus. J. Virol. **58:**233–237.
27. **Lucito, R., and R. J. Schneider.** 1992. Hepatitis B virus X protein activates transcription factor NF-κB without a requirement for protein kinase C. J. Virol. **66:**983–991.
28. **Martin, J. M., D. Veis, S. J. Korsmeyer, and B. Sugden.** Latent membrane protein of Epstein-Barr virus induces cellular phenotypes independently of expression of Bcl-2. J. Virol. **67:**5269–5278.

28a. **McElhinney, J. A., W. S. MacMorran, G. D. Bren, R. M. Ten, A. Israël, and C. V. Paya.** 1995. Regulation of IκBα and p105 in monocytes and macrophages persistently infected with human immunodeficiency virus. J. Virol. **69:**1500–1509.

29. **Mellits, K. H., R. T. Hay, and S. Goodbourn.** 1993. Proteolytic degradation of MAD3 (IκBα) and enhanced processing of the NF-κB precursor p105 are obligatory steps in the activation of NF-κB. Nucleic Acids Res. **21:**5059–5066.
30. **Nalesnik, M. A.** 1991. Lymphoproliferative disease in organ transplant recipients. Semin. Immunopathol. **13:**199–216.
31. **Nolan, G. P., and D. Baltimore.** 1992. The inhibitory ankyrin and activator rel proteins. Curr. Opin. Genet. Dev. **2:**211–220.
32. **Petti, L., and D. DiMaio.** 1994. Specific interaction between the bovine papillomavirus E5 transforming protein and the beta receptor for platelet-derived growth factor in stably transformed and acutely transfected cells. J. Virol. **68:**3582–3592.
33. **Rice, N. R., and M. K. Ernst.** 1993. *In vivo* control of NF-κB activation by IκBα. EMBO J. **12:**4685–4695.
34. **Rowe, M., M. Peng-Pilon, D. S. Huen, R. Hardy, D. Croom-Carter, E. Lundgren, and A. B. Rickinson.** 1994. Upregulation of *bcl-2* by the Epstein-Barr virus latent membrane protein LMP1: a B-cell-specific response that is delayed relative to NF-κB activation and to induction of cell surface markers. J. Virol. **68:**5602–5612.
35. **Schmid, R. M., N. D. Perkins, C. S. Duckett, P. C. Andrews, and G. J. Nabel.** 1991. Cloning of an NF-κB subunit which stimulates HIV transcription in synergy with p65. Nature (London) **352:**733–736.

35a. **Steffan, N., and C. Paya.** Unpublished data.

36. **Straus, S. E., J. I. Cohen, G. Tosato, and J. Meier.** 1993. Epstein-Barr virus infections: biology, pathogenesis, and management. Ann. Intern. Med. **118:** 45–58.
37. **Sun, S.-C., J. Elwood, C. Beraud, and W. C. Greene.** 1994. Human T-cell leukemia virus type I tax activation of NF-κB/Rel involves phosphorylation to degradation of IκBα and RelA (p65)-mediated induction of the c-*rel* gene. Mol. Cell. Biol. **14:**7377–7384.
38. **Sun, S.-C., P. A. Ganchi, D. W. Ballard, and W. C. Greene.** 1993. NF-κB controls expression of inhibitor IκBα. Science **259:**1912–1915.
39. **Sun, S-C, P. A. Ganchi, C. Beraud, D. W. Ballard, and W. C. Greene.** 1994. Autoregulation of the NF-κB transactivator RelA (p65) by multiple cytoplasmic inhibitors containing ankyrin motifs. Proc. Natl. Acad. Sci. USA **91:**1346–1350.
40. **Ten, R. M., C. V. Paya, N. Israël, O. Le Bail, M.-G. Mattei, J.-L. Virelizier, P. Kourilsky, and A. Israël.** 1992. The characterization of the promoter of the gene encoding the p50 subunit of NF-κB indicates that it participates in its own regulation. EMBO J. **11:**195–203.
41. **Wang, D., D. Liebowitz, and E. Kieff.** 1985. An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells. Cell **43:**831–840.
42. **Wang, F., C. Gregory, C. Sample, M. Rowe, D. Liebowitz, R. Murray, A. Rickinson, and E. Kieff.** 1990. Epstein-Barr virus latent membrane protein (LMP1) and nuclear proteins 2 and 3C are effectors of phenotypic changes in B lymphocytes: EBNA-2 and LMP1 cooperatively induce CD23. J. Virol. **64:**2309–2318.
43. **Young, L. S., and M. Rowe.** 1992. Epstein-Barr virus, lymphomas and Hodgkin's disease. Semin. Cancer Biol. **3:**273–284.
44. **Zabel, U., and P. A. Baeuerle.** 1990. Purified human IκB can rapidly dissociate the complex of the NF-κB transcription factor with its cognate DNA. Cell **61:**255–265.