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Stimulation of NF-κB-Mediated Transcription by Mutant Derivatives of the Latent Membrane Protein of Epstein-Barr Virus

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The latent membrane protein (LMP) of Epstein-Barr virus contributes to the immortalizing activity of the virus in primary, human B lymphocytes, but its mechanism of function is unknown. LMP is expressed at the plasma membrane and may act by influencing the signalling pathways of infected cells. LMP increases transcription of reporter plasmids that are responsive to members of the NF-κB/Rel family of transcription factors (M.-L. Hammarskjold and M. C. Simurda, J. Virol. 66:6496–6501, 1992, and A. Krikos, C. D. Laherty, and V. M. Dixit, J. Biol. Chem. 267:17971–17976, 1992). We measured the stimulation of the activity of a reporter plasmid by LMP in Jurkat and 293 cells in transfection experiments. Expression of LMP stimulated plasmids that contained κB enhancer elements but not plasmids that lacked the elements. In 293 cells, expression of the NF-κB inhibitor, IκB-α, reduced the stimulatory activity of LMP. We used deletional analysis to map the domains of LMP that are required for its activity in 293 cells. Wild-type LMP stimulated NF-κB by a factor of 20 to 30, while mutant derivatives of LMP that lack oncogenic activity stimulated NF-κB by a factor of 3. The multiple membrane-spanning segments together with the carboxy-terminal 55 amino acid residues of LMP were required for its maximal stimulatory function. Residues within its cytoplasmic amino terminus were not required for LMP's stimulation of NF-κB. We tested also for stimulation of NF-κB activity in cell lines known to support phenotypic changes mediated by expression of LMP. LMP stimulated little NF-κB activity in HEp2 cells and no detectable NF-κB activity in BALB/3T3 cells. The LMP stimulation of NF-κB factors that occurs in some cell lines provides a useful and biochemically tractable assay for determining the function of LMP.

Epstein-Barr virus (EBV) establishes a latent infection in human B lymphocytes by initiating and maintaining the proliferation of host cells, many of which become immortalized (26, 46). The latent membrane protein (LMP) of EBV contributes to this immortalizing activity (25), but its mechanism of function is unknown. LMP has oncogenic properties which show that it can influence the proliferation of lymphoid and nonlymphoid cells: it acts as an oncoprotein in selected, rodent fibroblastoid cell lines (3, 12, 41, 54), it may contribute to nasopharyngeal carcinogenesis because it is frequently expressed in tumor cells (11, 15), and it inhibits the differentiation of epithelial cell lines (16, 23, 58). LMP is also cytotoxic when overexpressed in a variety of cell lines, but its nononcogenic, mutant derivatives are not (20). Thus, the biochemical function of LMP in cells infected by EBV may be to influence cellular mechanisms that are essential for survival and proliferation and that operate in many cell types.

Several properties of LMP indicate that it may stimulate signal-transducing pathways in cells. LMP is an integral membrane protein with a cytoplasmic amino terminus of 25 residues, six membrane-spanning segments, and a cytoplasmic carboxy terminus of 200 residues (6, 17, 33). The attachment of LMP to the cytoskeleton, localization to patches in the plasma membrane, and its short half-life (2, 33, 36) correlate with the oncogenic activity of LMP (37) and are properties that are reminiscent of activated growth factor receptors. The carboxy terminus of LMP is phosphorylated on serine and threonine residues and is proteolytically cleaved during the turnover of LMP (2, 36, 40), but these properties are dissociable from its oncogenic function (4, 37, 41).

Signalling pathways are expected to induce expression of cellular proteins soon after being activated. Increased expression of some cellular proteins correlates with long-term expression of LMP (22, 29, 42, 55, 56), although such correlations may reflect selection for cells that can tolerate the cytotoxic activity of LMP. Previous experiments that measure the consequences of short-term expression of LMP (38, 43, 44, 48), together with those that study stable expression of LMP, indicate that LMP induces activation antigens, adhesion factors, and proteins which contribute to cellular survival in selected cell lines. The clearest indication that LMP stimulates signal-transducing pathways in cells is that the activity of NF-κB/Rel transcription factors is increased soon after LMP is expressed (21, 29, 48).

NF-κB factors might mediate several of the phenotypes associated with LMP. There are five known members of the mammalian NF-κB/Rel family: NFKB1, NFKB2, c-Rel, RelA, and RelB (reviewed in references 7 and 35). They form heterodimers with one another and are retained in the cytoplasm as inactive complexes by IκB inhibitors until stimulating agents induce the release of the inhibitors. Active NF-κB heterodimers increase transcription of genes from promoter sequences that contain κB elements. At least one of the genes induced by LMP, ICAM-1, is also expressed after stimulation of NF-κB factors (53).

If NF-κB factors were to mediate any of the phenotypes that are associated with LMP expression, then the mechanism by which their DNA-binding activities are activated might be closely linked to the mechanism of LMP function. It is useful

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therefore to determine whether stimulation of NF-κB activity is correlated with the activities of LMP. Stimulation of transcription by LMP was tested previously in transfection experiments using plasmids that were capable of run-away replication in mammalian cells (21) or using reporter plasmids that contained or lacked κB elements in the context of a complex promoter in one cell line in one set of experiments (29). Because NF-κB-mediated transcription can be affected by other factors that bind within or near κB elements (45, 52), it is informative to demonstrate that LMP stimulates NF-κB and not other factors.

We tested for the stimulation of NF-κB activity by LMP by using several reporter plasmids and by determining the effects of an NF-κB-inhibiting factor, IκB-α. One reporter plasmid that we used extensively contains only κB elements, along with the minimal sequences necessary for initiation of transcription, and binds a wide variety of NF-κB/rel dimers. This reporter plasmid and LMP were introduced by transfection into two cell lines that have previously been used to study NF-κB activity, Jurkat and 293 cells, and in two other cell lines that show phenotypes dependent on LMP expression, HEp2 and BALB/3T3 cells. We found that NF-κB was stimulated by LMP to different levels in 293, Jurkat, and HEp2 cells, but we detected no stimulation of NF-κB by LMP in BALB/3T3 cells. We used the 293 cell line to map the domains of LMP that are required for its stimulation of NF-κB and compared them to the domains required for other activities of LMP. We conclude that the stimulation of NF-κB-mediated transcription in appropriate cell lines provides a useful measure of one activity of LMP.

MATERIALS AND METHODS

Cells. The murine BALB/3T3 and human Jurkat, 293, and HEp2 cell lines were obtained from the American Type Culture Collection. BALB/3T3 cells (ATCC CCL 163) are endothelial cells (10a) and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (CS; Hyclone Laboratories, Logan, Utah). Jurkat clone E6-1 (ATCC TIB 152) is a T lymphoid and was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). 293 cells (ATCC CRL 1573) are transformed fibroblastoid cells established from embryonal kidneys, and were cultured in DMEM supplemented with 10% FBS. The HEp2 epithelial carcinoma cell line (ATCC CCL 23) was cultured in DMEM supplemented with 10% FBS. B95-8 is a marmoset cell line established by infection with EBV (39) and was cultured in RPMI 1640 supplemented with 10% CS. All media contained 200 U of penicillin per ml and 200 μg of streptomycin per ml. Cells were cultured at 37°C in ~5% atmospheric CO_2.

Plasmids. Each reporter plasmid contains the gene for luciferase under the transcriptional regulation of selected promoter and enhancer elements. The plasmid TARluc contains the long terminal repeat of human immunodeficiency virus type 1 (27). The plasmids A20L and dmA20L have the promoter of the gene encoding A20 (29) or a promoter altered by mutation of each of two κB elements, respectively. These plasmids were constructed by removing the CAT (chloramphenicol acetyltransferase) genes from A20CAT and dmA20CAT (29) by digestion with *Sal*I and *Bam*HI and replacing them with a 2.7-kbp *Xho*I-*Bam*HI fragment containing the luciferase gene from pGL2-Basic (Promega, Madison, Wis.). The plasmids 3X-κB-L and 3X-mutκ-L have a minimal *fos* promoter element and three copies of either the major histocompatibility complex (MHC) class I κB element TGGGGATTCCCCA or its altered counterpart TGcGGATTCCCgA (5), with lowercase letters denoting base changes relative to the wild-type sequence. They were constructed by ligation of ~0.2-kbp fragments obtained from MHC-NF-κB CAT or MHCmut-NF-κB CAT (5) by digestion with *Hin*dIII, blunting with Klenow DNA polymerase, and then digestion with *Bam*HI. The fragments were ligated to pGL2-Basic that had been digested with *Sma*I and *Bgl*II.

LMP and all but one of its derivatives, LMP-NΔ25, were expressed from plasmids derived from pSV_2BNLF-1 (2), which has EBV B95-8 sequences 169566 to 167129 (1) in place of those encoding CAT of the vector plasmid SV_2CAT (19). The SV_2BNLF-1 plasmid will hereafter be called pSV_2LMP, and its derivatives will be designated either by the number of N- or C-terminal residues deleted or by the positions of amino acid residues internal to the LMP that were deleted. The plasmids SV_2LMP-NΔ43, -CΔ155, -CΔ174, -CΔ199 (4), -Δ25-132 (20), and -CΔ55 (37) have been described previously. LMP-NΔ25 was expressed from a plasmid, pCMV-NΔ25 (37), containing the immediate-early promoter and enhancer of human cytomegalovirus (CMV). The plasmids used to express other gene products from the immediate-early human CMV promoter/enhancer element have been described previously: pCMV-IκB-α (14), pCMV-NFKB1-RelA (formerly p50/65 [30]), and EQ176 (called here pCMV-βGal [51]).

The plasmid SV_2LMP-Δ188-331 was derived from pSV_2LMP by deleting sequences between the *Nco*I site at the codon for residue 187 and the *Bst*EII site. The Klenow fragment of *Escherichia coli* DNA polymerase was used to repair sequences after digestion with *Nco*I and *Bst*EII so that the translational reading frame was preserved at the site of the deletion. The plasmid SV_2LMP-Δ231-331 was made by deleting sequences between the *Ngo*MI and *Bst*EII sites of pSV_2LMP. The translational reading frame was preserved by using mung bean nuclease after cleavage with *Ngo*MI and Klenow polymerase after cleavage with *Bst*EII to excise or repair single-stranded sequences, respectively. The plasmid SV_2LMP-Δ213-232 was made by deleting sequences between the *Msc*I and *Ngo*MI sites of pSV_2LMP. Single-stranded sequences left at the site of cleavage by *Ngo*MI were excised with mung bean nuclease to preserve the reading frame.

The plasmid SV_2LMP-Δ12-20 was constructed by replacing sequences of pSV_2LMP between the *Sfi*I and *Stu*I sites of the amino-terminal coding region with those of an oligonucleotide that lacked codons for residues 12 to 20 of LMP. The plasmid SV_2LMP-PA15,16 was made by replacing sequences of pSV_2LMP between the *Sfi*I and *Xho*I sites with an oligonucleotide specifying translation of alanine, rather than proline, at the 15th and 16th codons of LMP. The plasmid SV_2LMP-P9,10,15 was made by using a DNA fragment from a PCR in which sequences between and including the *Mlu*I and *Xho*I sites of pSV_2LMP were amplified from DNA primers that specified translation of alanine rather than proline at the 9th and 10th codons of LMP. The reaction product also contained a sequence change that coded for histidine rather than proline at position 15. The amplified reaction product was digested with *Mlu*I and *Xho*I and ligated to pSV_2LMP at the corresponding sites of cleavage.

Recombinant DNA manipulations were performed with enzymes as recommended by the supplier (New England Biolabs, Beverly, Mass.). PCRs were performed with reagents supplied by Boehringer Mannheim Biochemicals (Indianapolis, Ind.), by using a DNA thermal cycler 480 (Perkin Elmer, Norwalk, Conn.). Oligonucleotides were purchased from Genosys (The Woodlands, Tex.). Portions of plasmids encoding mutant derivatives of LMP were sequenced to ensure that the expected sequences were deleted and that translational reading frames were preserved at the site of deletion or that introduction of oligonucleotides and PCR products introduced the expected changes. Plasmids were sequenced by using the Sequenase version 2.0 reagent kit as recommended by its manufacturer (United States Biochemicals, Cleveland, Ohio). Plasmid DNAs were extracted by a modification of the alkaline lysis method (9) and then banded twice in gradients of cesium chloride (49).

Transfections. The amounts of plasmid DNAs that were transfected are reported in the figure legends. These amounts were determined to yield equal levels of expression of LMP and its derivatives whenever possible, by measuring that expression in Western blots (immunoblots). Generally, 10 μg of each of the plasmids that express LMP and its derivatives, except pSV_2-LMP-NΔ43 and pCMV-LMP-NΔ25, was transfected in electroporation experiments and produced similar levels of expression of the detected LMP derivatives. Transfection of pSV_2-LMP-NΔ43 in 293 and HEp2 cells required 50% more DNA to produce levels of expression that were equivalent to those of wild-type LMP. Expression of similar levels of LMP-NΔ25 required that 0.2 μg of pCMV-LMP-NΔ25 be transfected in 293 cells.

Because the promoters contained in each expression construct might titrate transcription factors in transfected cells and in themselves affect the activities of the reporter plasmids, every transfection in an experiment included identical amounts of plasmid DNAs that contained a simian virus 40 early promoter or a CMV immediate-early promoter. The plasmid SV_2CAT was used as a control for pSV_2LMP and for the plasmids that express LMP derivatives from the simian virus 40 early promoter, and pCMV-βGal was used as control for pCMV-NΔ25, -IκB-α, and -NFKB1-RelA.

Jurkat, HEp2, and 293 cells were transfected by electroporation with a University of Wisconsin—Madison electroporator (28). Jurkat cells were resuspended at 4×10^7 cells per ml in RPMI with 10% FBS and 0.05 M HEPES buffer (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.4). HEp2 cells were trypsinized, rinsed in culture medium, and resuspended at 5×10^6 cells per ml in DMEM with 10% CS and 0.05 M HEPES (pH 7.4). 293 cells were trypsinized, rinsed in culture medium, and resuspended at 2×10^7 cells per ml in DMEM with 10% FBS and 0.05 M HEPES (pH 7.4). Portions of resuspended cells (1.1 ml) were chilled on ice, mixed with plasmid DNA, and then transferred in 0.25-ml aliquots to each of four 0.4-cm Gene Pulser cuvettes (Bio-Rad Laboratories, Richmond, Calif.). Jurkat cells were electroporated such that the capacitance discharge produced a waveform with a V_{max} of 5 and τ of 30 ms. Electroporation of HEp2 cells was performed with a waveform showing a V_{max} of 6 and τ of 18 ms. Electroporation of 293 cells was performed with a V_{max} of 3 and τ of 35 ms. After electroporation, the cells from each of two cuvettes were pooled into one tissue culture dish in the culture medium appropriate for each cell line. Jurkat and 293 cells were scored 40 to 42 h after electroporation, and HEp2 cells were tested 24 h after electroporation.

BALB/3T3 cells were transfected by the calcium phosphate precipitation method (49). BALB/3T3 cells, 4×10^5 per 100-cm culture dish, were cultured for 2 days. DNAs were precipitated at a concentration of 40 μg of DNA per ml of solution, and then 0.5 ml of the suspension containing precipitated DNA was

TABLE 1. Stimulation of reporter plasmid activity by LMP in 293 and Jurkat cells[a]

Plasmid	293		Jurkat	
	Basal activity (10^5 RLU/ 10^6 cells)[b]	Fold stimulation by LMP[c]	Basal activity (10^5 RLU/ 10^6 cells)	Fold stimulation by LMP
NF-κB-responsive reporter				
TARluc	10	5	0.2	10
A20L	1.8	4	0.25	6
3X-κB-L	1.4	18	0.26	11
Mutant reporter				
dmA20L	0.3	0.7	0.9	0.9
3X-mutκ-L	0.1	0.9	0.1	1.2

[a] 293 or Jurkat cells were electroporated with 5 μg of reporter plasmid and 7.5 μg of pSV_2LMP or pSV_2CAT and tested for luciferase activity after 40 to 42 h. Each pair of plasmids transfected was tested in duplicate in each experiment. The results shown are averages from two independent experiments.

[b] Basal luciferase activity was determined after electroporation of reporter plasmid and pSV_2CAT. RLU, relative light units.

[c] Stimulation by LMP was calculated as the fold-stimulation of basal luciferase activity by dividing the relative light units observed after transfection of a given reporter plasmid and pSV_2LMP by the relative light units determined after transfection of that reporter plasmid and pSV_2CAT.

added to each of two culture dishes. DNA mixes were supplemented with pUC19 (New England Biolabs) to maintain uniform DNA concentrations. The cells were incubated with DNA precipitates for approximately 16 h, rinsed three times with DMEM, and then cultured in DMEM with 0.3 to 0.5% CS for 48 to 50 h. Some cell populations were treated with 20 nM phorbol 12-myristate 13-acetate (TPA; Sigma Chemical Co., St. Louis, Mo.) or with 10 ng of recombinant human tumor necrosis factor alpha (TNF-α; Promega) per ml of culture medium 16 h prior to scoring reporter plasmid activity.

Luciferase assay. Luciferase activity was measured in transfected cells by using reagents from the luciferase assay system (Promega). Cells were harvested, rinsed with phosphate-buffered saline (PBS), counted, pelleted, lysed (at cell densities of 10^8 Jurkat, 5×10^7 293, 10^7 HEp2, or 2×10^7 BALB/3T3 cells per ml of lysis reagent), and then centrifuged. Lysis supernatants, 20 μl each, were incubated with excess luciferase substrate, and the light emitted was detected by using a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, Calif.).

Western blot analysis. Cells were harvested, rinsed with PBS, counted, pelleted, and then lysed in 1× sodium dodecyl sulfate (SDS) sample buffer (49). The cellular lysates were sonicated and centrifuged to remove any aggregated material. Proteins from the lysates were resolved by SDS-polyacrylamide gel electrophoresis (31) and transferred to nitrocellulose membranes (34). The membranes were blocked with 0.3% casein (Tropix, Bedford, Mass.) in 0.2% Tween 20 detergent (Atlas Chemical Industries, Wilmington, Del.) and then incubated with affinity-purified, polyclonal rabbit antibodies specific for epitopes contained in the carboxy terminus of LMP. These antibodies were collected from rabbits after immunization with a recombinant, protein fusion of β-galactosidase and the last 199 amino-acid residues of LMP, as described previously (2). The blots were washed in PBS containing 0.2% Tween 20 and then incubated with a 1:1,000 dilution of anti-rabbit immunoglobulin G from sheep conjugated to alkaline phosphatase (Boehringer Mannheim Biochemicals). The blots were washed again and prepared for the chemiluminescent reaction with the substrate CSPD as recommended by its supplier (Tropix). Bound antibodies were visualized by chemiluminescent exposure of X-Omat AR film (Kodak, Rochester, N.Y.) for times ranging from 3 to 12 min.

RESULTS

LMP-stimulated reporter activity requires κB elements. We tested five reporter plasmids for their response to LMP expression in order to test the hypothesis that LMP stimulates transcriptional activity by activating members of the NF-κB family of transcription factors. Each of three reporter plasmids that contained κB elements showed greater activity in Jurkat and in 293 cells when the LMP expression vector pSV_2LMP was transfected, than when the control vector pSV_2CAT was transfected (Table 1). The plasmids TARluc and A20L each contain complex promoters with multiple, transcription factor-binding sites regulating expression of the reporter gene encoding luciferase. The plasmid 3X-κB-L contains three copies of the κB element of the murine MHC class I and the minimal promoter region of *fos* (5).

Two reporter plasmids that lack κB elements, dmA20L and 3X-mutκ-L, showed no response to LMP expression (Table 1). These plasmids differ from pA20L and p3X-κB-L, respectively, only by point mutation of their κB elements. The plasmid 3X-mutκ-L was not stimulated in cells in which LMP expression was confirmed (Fig. 1). Moreover, this plasmid was not stimulated by a chimeric fusion protein, NFKB1-RelA, which has the DNA-binding region of NFKB1 and the transactivating domain of RelA (30), and which stimulated strongly the activity of p3X-κB-L in 293 cells (Fig. 1A). Therefore, LMP-stimulated expression from p3X-κB-L and pA20L reporter plasmids required that they contain functional κB elements. This finding was consistent with the hypothesis that LMP stimulates transcription by activating NF-κB proteins but could not exclude the possibility that undefined transcription factors medi-

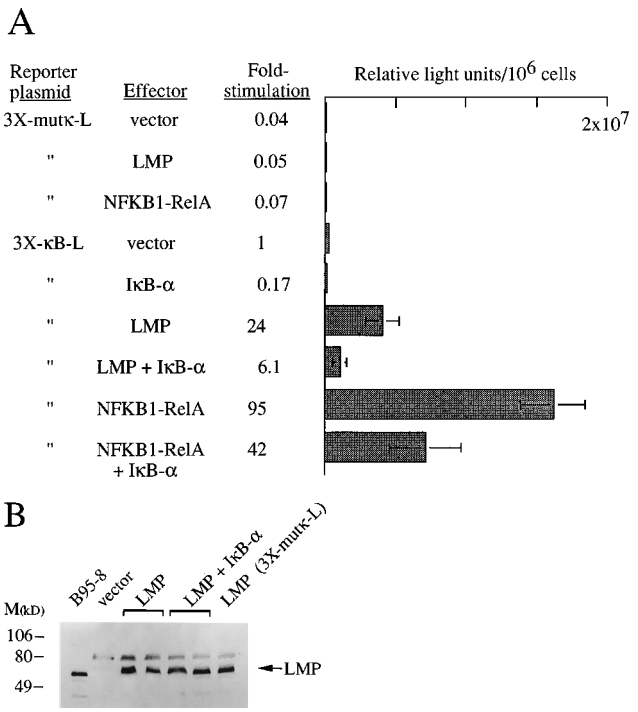

FIG. 1. Inhibition by IκB-α of LMP-stimulated reporter activity in 293 cells. 293 cells were transfected by electroporation and tested after 40 to 42 h for luciferase activity and expression of LMP. (A) Luciferase activity of reporter plasmids was determined after transfection of 5 μg of mutant or NFκB-responsive reporter plasmid and 10 μg of either pSV_2LMP or pSV_2CAT (vector) as a control. Every transfection included 2 μg of either pCMV-IκB-α to test for inhibition of NF-κB activity, pCMV-NFKB1-RelA to test for responsiveness of the reporter plasmids, or pCMV-β-Gal as control DNA. Fold-stimulation shows the reporter activity of each set of plasmid transfections as a value that was normalized to that observed after transfection of 3X-κB-L, SV_2CAT, and CMV-βGal. The graph shows luciferase activity plotted as relative light units detected in 10^6 cells, and error bars that indicate the standard deviation from the mean. The mean and standard deviation were calculated from four experiments, each of which was performed in duplicate for each set of plasmids tested. (B) Portions of the cell populations tested for luciferase activity for panel A were analyzed for LMP expression by Western blot analysis. Bound antibody was detected by chemiluminescent exposure of X-ray film. The lanes shown are LMP detected in protein lysates from 2×10^4 B95-8 EBV^+ cells or from 10^5 293 cells after transfection with either pSV_2CAT, pSV_2LMP (with duplicate lanes), pSV_2LMP and pCMV-IκB-α (with duplicate lanes), or pSV_2LMP and mutant reporter plasmid. The positions of molecular mass markers (M [in kilodaltons]) and of LMP are shown on the left and right, respectively.

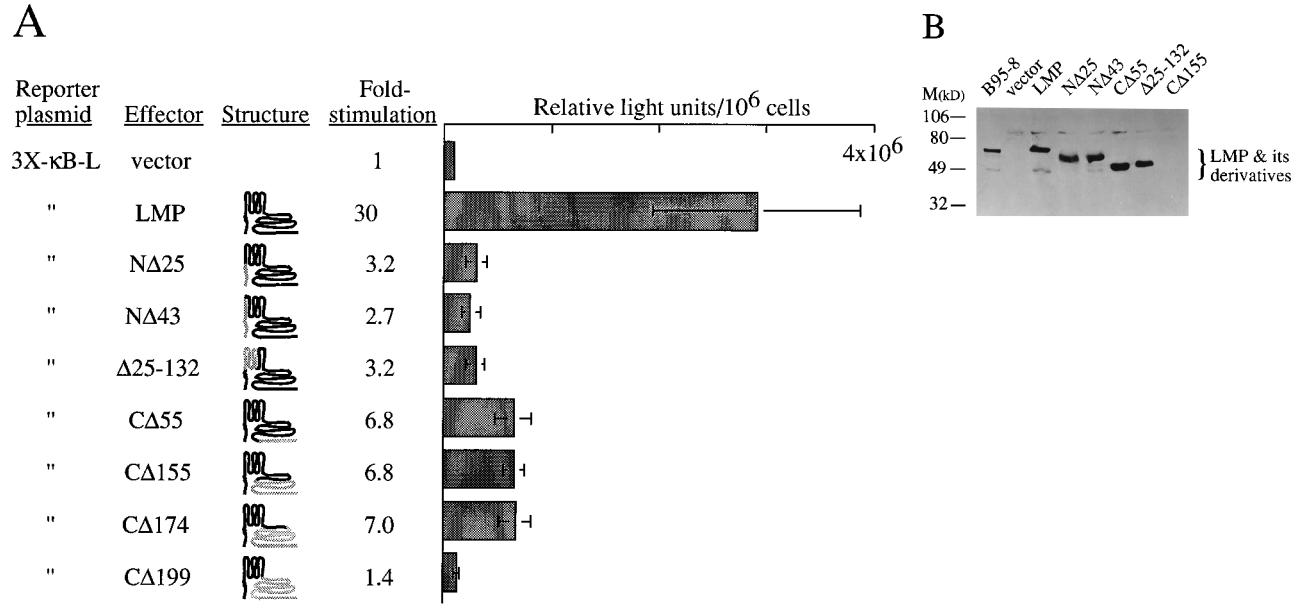

FIG. 2. Stimulation of NF-κB activity by LMP and its mutant derivatives in 293 cells. 293 cells were transfected by electroporation and tested after 40 to 42 h for luciferase activity and expression of LMP. (A) Plasmids, 10 μg of each, that express LMP or the indicated derivatives of LMP were transfected with 5 μg of 3X-κB-L reporter plasmid. Plasmids CMV-NΔ25 and SV_2LMP-NΔ43 were transfected in quantities of 0.2 and 15 μg, respectively, to ensure that cells expressing equivalent amounts of LMP derivatives were compared. The names and structures of the LMP derivatives are shown, with deleted regions indicated by lighter lines (structure). The fold-stimulation of NF-κB activity by LMP derivatives was calculated by comparison to the luciferase activity that was detected after transfection of pSV_2CAT (vector). The graph shows luciferase activity as relative light units detected in 10^6 cells. Error bars indicate the standard deviation of the mean. Derivatives LMP-NΔ25, -Δ25-132, -CΔ174, and -CΔ199 were tested in duplicate in three experiments. LMP-CΔ55 and -CΔ155 were tested in five experiments, and the remainder were tested in six experiments. (B) LMP expression was measured by Western blot analysis in portions of the same populations of transfected cells that were tested for panel A. Lanes shown contain LMP detected in protein lysates from 10^4 B95-8 EBV^+ cells or lysates from 2×10^5 293 cells after transfection with pSV_2CAT (vector), with pSV_2LMP, or with plasmids that express the indicated derivatives of LMP. The positions of molecular mass markers (M [in kilodaltons]) and the region of the blot that contains LMP and its derivatives are shown on the left and right, respectively.

ated the stimulation. Because transfection of 293 cells with 3X-κB-L and pSV_2LMP generated the greatest fold-stimulation of reporter plasmid activity of the plasmids tested, we used this cell line and plasmid to study further the stimulatory effects of LMP.

LMP-stimulated reporter activity is reduced by the NF-κB inhibitor, IκB-α. Additional evidence that LMP stimulates expression that is mediated by NF-κB was gained by testing the NF-κB-specific inhibitor, IκB-α, for its effect on LMP activity. Because NF-κB inhibitors act by retaining DNA-binding members of the NF-κB family in the cytoplasm, we predicted that overexpression of IκB-α would reduce stimulation by LMP, if NF-κB factors mediated its activity. 293 cells were transfected with p3X-κB-L and pSV_2LMP with and without pCMV-IκB-α. LMP when expressed alone stimulated reporter activity 24-fold but when expressed together with IκB-α stimulated reporter activity sixfold (Fig. 1A). Expression of IκB-α together with NFKB1-RelA reduced the stimulation of reporter plasmid to 44% of that with NFKB1-RelA alone, demonstrating that IκB-α can inhibit expression mediated by NF-κB proteins in this assay. Expression of IκB-α alone reduced the activity of p3X-κB-L to 17% of its basal level (Fig. 1A), indicating that this reporter plasmid depends on NF-κB factors for most of its activity.

The inhibition of LMP-stimulated activity by IκB-α could be interpreted to mean that LMP expression was reduced by IκB-α. We tested for LMP expression by Western blot analysis in portions of the cell populations that were used to detect activity of the reporter plasmid. The amount of LMP expressed in cells transfected with pSV_2LMP was equivalent to that of cells transfected with pSV_2LMP and pCMV-IκB-α (Fig. 1B). In other experiments, IκB-α had no effect on the activity of the early promoter of simian virus 40, the promoter element used to express LMP from pSV_2LMP, as determined by measuring the expression of luciferase from a plasmid containing this promoter (data not shown). IκB-α therefore inhibited activity induced by LMP, rather than the expression of LMP.

The results shown in Fig. 1 indicated that IκB-α could inhibit 75 to 80% of the detected activity of NF-κB factors present constitutively in 293 cells. IκB-α inhibited LMP-dependent activity by a similar degree. These findings indicated that NF-κB factors are required for the stimulation of transcription by LMP.

Stimulation of NF-κB activity by mutant derivatives of LMP. Mutant derivatives of LMP have previously been used to show that some of its structural domains are required for oncogenic activity. We tested several mutant derivatives of LMP in order to determine which of its structural domains are required for the stimulation of NF-κB activity. We first tested a set of derivatives of LMP that were constructed to permit analysis of each of its three major structural domains: the cytoplasmic amino terminus, the membrane-spanning domains, and the cytoplasmic carboxy terminus. Luciferase activity was measured in 293 cells as a measure of NF-κB stimulation after the cells were transfected with p3X-κB-L and with one of several plasmids expressing different mutant derivatives of LMP. Each of the mutant derivatives of LMP stimulated less reporter activity than that stimulated by wild-type LMP.

The mutant derivatives LMP-NΔ25 and LMP-NΔ43 lack the amino-terminal 25 and 43 amino acid residues of LMP, respectively. Each of these derivatives stimulated 10% of the NF-κB activity that was stimulated by wild-type LMP, showing that the

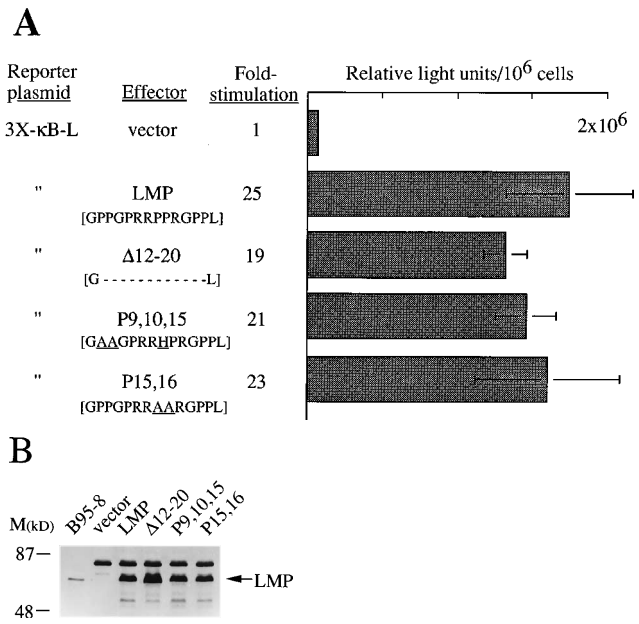

FIG. 3. Stimulation of NF-κB activity by derivatives of LMP with altered N-terminal sequences. 293 cells were transfected by electroporation and tested after 40 to 42 h for luciferase activity and expression of LMP. (A) Plasmids, 10 μg each, that express LMP or the indicated derivatives of LMP were transfected together with 5 μg of 3X-κB-L reporter plasmid. Amino acid residues 8 to 21 of the wild-type LMP and the sequences of LMP mutant derivatives are shown. Deleted residues are indicated by a hyphen, and substituted residues are underlined. The fold-stimulation of NF-κB activity by LMP derivatives was calculated by comparison to the luciferase activity detected after transfection of reporter plasmid and pSV_2CAT (vector). The graph shows luciferase activity as relative light units detected in 10^6 cells. The standard deviation of mean luciferase activity is designated by error bars and was calculated from duplicate measurements made in each of three experiments. (B) LMP expression was measured by Western blot analysis of portions of the same populations of transfected cells that were tested for panel A. Lanes shown are LMP detected in protein lysates from 10^4 B95-8 EBV^+ cells or in lysates from 2×10^5 293 cells after transfection with pSV_2CAT (vector), with pSV_2LMP, or with plasmids that express the indicated derivatives of LMP. The positions of molecular mass markers (M [in kilodaltons]) and the region of the blot that contains LMP and its derivatives are indicated on the left and right, respectively.

amino terminus was required for LMP to function in this assay above minimal levels. The derivative LMP-Δ25-132 lacks the first four of six transmembrane domains but retains both the amino and carboxy termini. It too stimulated minimal NF-κB transcription, showing 10% of the activity of wild-type. Each of these three derivatives, LMP-NΔ25, -NΔ43, and Δ25-132, was expressed at levels equivalent to that of wild-type LMP (Fig. 2B).

The mutant derivatives LMP-CΔ55, -CΔ155, -CΔ174, and -CΔ199 have progressive truncations of the carboxy terminus. Each of these derivatives, except LMP-CΔ199, showed approximately 25% of the NF-κB-stimulating activity of wild-type LMP. Although lower than the wild-type level, this level of stimulation was reproducibly greater than that observed with the minimally active derivatives LMP-NΔ25, -NΔ43, and -Δ25-132. Western blot analysis demonstrated that LMP-CΔ55 was expressed at levels equivalent to that of wild-type LMP (Fig. 2B). The remaining derivatives could not be detected, because epitopes recognized by the polyclonal antibodies used to detect LMP have presumably been removed. The LMP-specific, polyclonal antibodies used to detect LMP were produced after immunization with a fusion protein containing the last 199 amino acid residues of LMP (2). The major epitopes within this region of LMP have not been identified. We inferred that the activity of the derivatives CΔ55, CΔ155, and CΔ174 were similar to one another because an equivalent amount of plasmid encoding each derivative was transfected into cells and showed equivalent activities. Each of the plasmids has been shown previously to induce anchorage-independent proliferation when used to transfect BALB/3T3 cells (4, 37). Moreover, LMP-CΔ155 and LMP-CΔ174 can be detected when expressed at high levels in Cos cells (4).

LMP-CΔ199 stimulated no NF-κB activity, relative to the vector control. This derivative encodes Met-Tyr-Tyr-His-Ala-Ser-Leu-Asp-stop at its carboxy terminus, with the Met residue forming the approximate boundary between the sixth membrane-spanning segment of LMP and its cytoplasmic tail. The corresponding sequence of wild-type LMP begins Met-Tyr-Tyr-His-Gly-Gln-Arg-His-Ser-Asp. It is unclear whether the cytoplasmic residues that remain in LMP-CΔ199 are sufficient to stabilize the adjacent membrane-spanning region, and because we cannot detect this derivative we were unable to identify the cause for its failure to stimulate NF-κB.

The impaired activity of the mutant derivatives whose expression could be confirmed, LMP-NΔ25, -NΔ43, -Δ25-132, and -CΔ55, proved that each of the three major structural domains of LMP contributes to its stimulation of NF-κB activity in 293 cells.

Stimulation of NF-κB activity by mutant derivatives of LMP with altered amino termini. The cytoplasmic amino terminus of LMP has proline residues in a configuration similar to those of SH3-binding domains, which mediate protein-protein interactions (see Discussion). The amino terminus of LMP shows two possible matches to the extended SH3-binding motif proposed by Yu et al. (57) and three possible matches to the limited motif PXXP. Two sequences similar to the extended consensus begin at residues 6 and 13 of LMP. Three mutant derivatives of LMP that alter part or all of the potential SH3-binding domains in this region of LMP were constructed and tested in 293 cells for stimulation of NF-κB activity (Fig. 3).

Each of the three amino-terminal mutants of LMP showed NF-κB-stimulating activity that was indistinguishable from that of wild-type LMP (Fig. 3A), and each of the derivatives was expressed at levels equivalent to that of the wild type (Fig. 3B). A proline-rich, SH3-binding motif found in the amino-terminal domain, therefore, does not contribute to the NF-κB-stimulating activity of LMP.

Stimulation of NF-κB activity by derivatives of LMP with altered carboxy termini. The behavior of the derivatives shown in Fig. 2 revealed that the carboxy terminus contributed to the function of LMP in 293 cells. The similar levels of activity of LMP-CΔ55, -CΔ155, and -CΔ174 prompted two hypotheses: either a domain required for maximal stimulation of NF-κB activity is restricted to the last 55 residues of LMP, or a domain required for maximal activity is specified by a region that is larger than but includes the last 55 residues. Also, LMP-CΔ199 failed to stimulate the activity of NF-κB, a result which seemed to indicate that LMP has an additional region important for its function within the 25 amino acid residues of the carboxy terminus that are adjacent to the membrane.

We found by testing three other derivatives of LMP that its carboxy terminus is likely to contain only one region required for maximal stimulation of NF-κB activity and that this region lies within the last 55 carboxy-terminal residues. Each of the three derivatives lacks different amino-acid residues internal to the carboxy terminus, but each of them retains at least the last 55 residues. These LMP derivatives stimulated NF-κB activity in 293 cells similarly to the wild-type LMP (Fig. 4A). Only LMP-Δ213-232 could be detected by Western blot analysis (Fig. 4B). The epitopes recognized by the antibodies that were

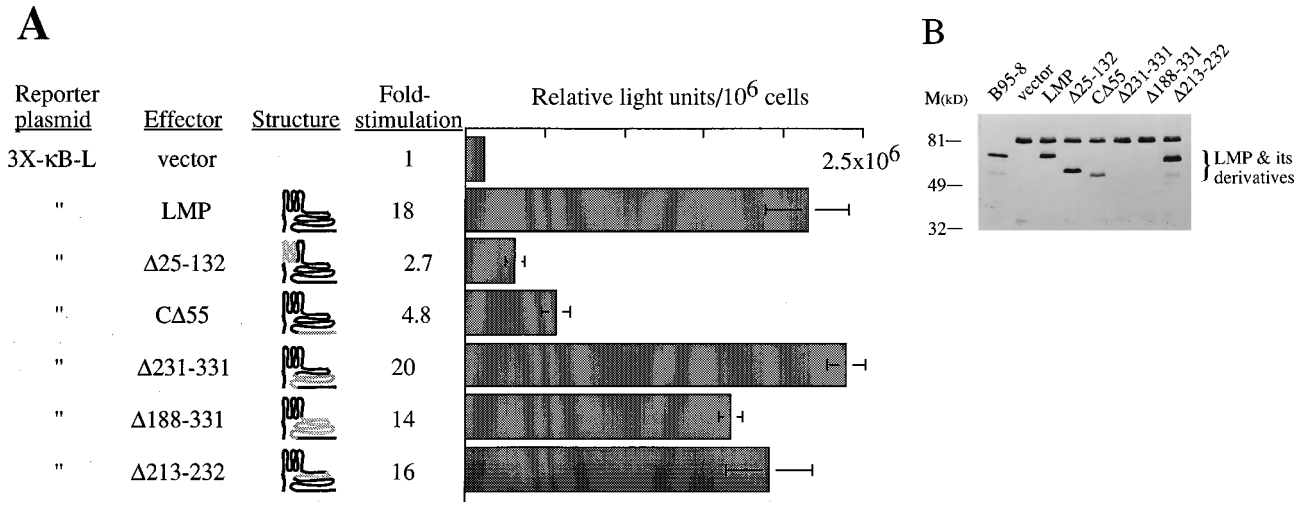

FIG. 4. Stimulation of NF-κB by LMP derivatives that have altered C-terminal sequences. 293 cells were transfected by electroporation and tested after 40 to 42 h for luciferase activity and expression of LMP. (A) Plasmids, 10 μg of each, that express LMP or the indicated derivatives of LMP were transfected together with 5 μg of 3X-κB-L reporter plasmid. The structures of the LMP derivatives are shown, with deleted regions indicated by lighter lines (structure). The fold-stimulation of NFκB activity by LMP derivatives was calculated by comparison to the luciferase activity that was detected after transfection of reporter plasmid and pSV_2CAT (vector). The graph shows luciferase activity as the relative light units detected in 10^6 cells. The standard deviation of mean luciferase activity is designated by error bars and was calculated from duplicate measurements made in each of three experiments. (B) LMP expression was measured by Western blot analysis of portions of the same populations of transfected cells that were tested for panel A. Lanes shown are LMP detected in protein lysates from 10^4 B95-8 EBV^+ cells or in lysates from 2×10^5 293 cells after transfection with pSV_2CAT (vector), with pSV_2LMP, or with plasmids that express the indicated derivatives of LMP. The positions of molecular mass markers (M [in kilodaltons]) and the region of the gel containing LMP and its derivatives are indicated on the left and right, respectively.

used to detect LMP therefore appear to be specified by some portion of residues 233 to 330 of LMP. These results proved that the residues missing from LMP-Δ213-232 do not contribute to the NF-κB-stimulating activity of LMP in 293 cells and were consistent with the hypothesis that the only region of the carboxy terminus of LMP that is required for its maximal stimulation of NF-κB transcription is contained within the last 55 residues.

NF-κB activity in HEp2 and BALB/3T3 cells. LMP is cytotoxic when overexpressed in HEp2 and BALB/3T3 cells (20) and is oncogenic when expressed at low levels in BALB/3T3 cells (3). If phenotypes associated with LMP expression, such

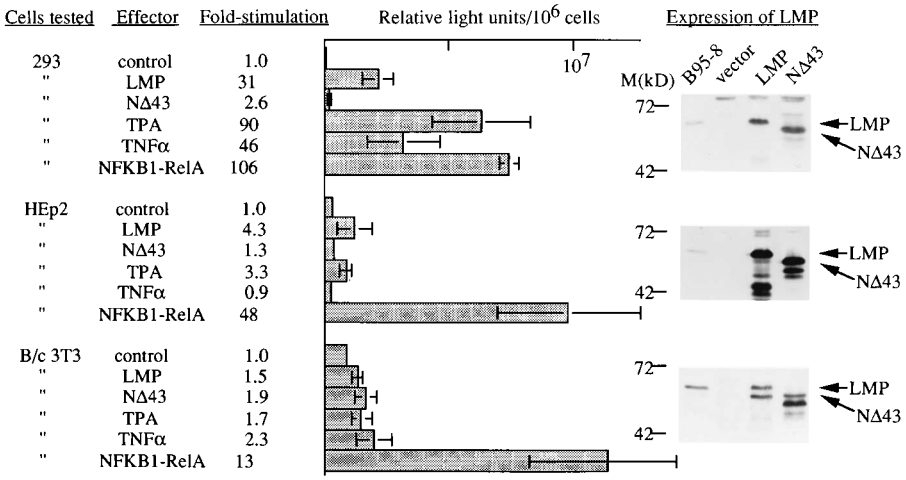

FIG. 5. Stimulation of NF-κB activity by LMP, TPA, or TNF-α in 293, HEp2, and BALB/3T3 cells. 293 and HEp2 were transfected by electroporation with 5 μg of p3X-κB-L and with either 10 μg of pSV_2CAT, 10 μg of pSV_2LMP, or 15 μg of pSV_2LMP-NΔ43, to ensure that cells expressing equivalent amounts of LMP and LMP-ND43 were compared. All transfections included 1 μg of pCMV-βGal, except the samples labelled NFKB-RelA, which included 1 μg of pCMV-NFKB1-RelA. BALB/3T3 cells were transfected by the calcium phosphate method with 5 μg of p3X-κB-L, together with 0.5 μg of either pSV_2CAT, pSV_2LMP, or pSV_2LMP-NΔ43, and with 0.3 μg of either pCMV-βGal or pCMV-NFKB1-RelA. Some cell samples were transfected with reporter plasmid and control vectors and then treated with TPA or TNF-α. Fold-stimulation of NF-κB was determined by the comparison to the luciferase activity detected after transfection of the reporter plasmid, pSV_2CAT, and pCMV-βGal (control). Luciferase activity is plotted in the graph as relative light units detected in 10^6 cells. The standard deviation of mean luciferase activity is indicated by error bars for those samples that produced significantly more activity than the control. The mean and standard deviation were calculated from duplicate measurements made in two (293 cells) or three (HEp2 and BALB/3T3 cells) experiments. Expression of LMP and LMP-NΔ43 in the cells tested for luciferase activity is shown on the right. The lanes in each blot show LMP detected in protein lysates from 10^4 B95-8 EBV^+ cells or from cells transfected with pSV_2CAT (vector), with pSV_2LMP, or with pSV_2LMP-NΔ43. Protein lysates were prepared from 10^5 293 and BALB/3T3 cells and from 10^4 HEp2 cells. The positions of molecular mass markers (M [in kilodaltons]) and the positions of LMP and of LMP-NΔ43 are indicated on the left and right, respectively.

TABLE 2. Properties of selected mutant derivatives of LMP

Property	Relative activity[a]				Reference
	LMP	LMP-NΔ43	LMP-Δ25-132	LMP-CΔ55	
Stimulation of NF-κB	++++	+	+	++	This study
Cytoskeletal attachment	++++	−	−	++	37
Turnover	++++	+	+	++	37
Patching at plasma membrane	++++	−	−	++++	37
Transformation of BALB/3T3 cells	++++	−	−	++++	4, 37
Cytotoxicity	++++	−	−	++++	20, 37

[a] Symbols indicate activities relative to that of the wild type: ++++, indistinguishable from wild-type activity; ++, intermediate to wild-type activity; +, less than intermediate activity but above background; −, indistinguishable from background.

as cytotoxicity and oncogenic transformation, were mediated by NF-κB factors, then NF-κB activity in cells should increase when LMP is introduced. NF-κB activity was increased moderately in HEp2 cells but was not increased detectably in BALB/3T3 cells after expression of LMP.

HEp2, BALB/3T3, and 293 cells were transfected with the 3X-κB-L reporter plasmid, together with pSV_2CAT, pSV_2LMP, or pSV_2LMP-NΔ43. Some cells were transfected with reporter plasmid and pCMV-NFKB1-RelA, or its control vector pCMV-βGal, to assess the function of a selected NF-κB factor in the cells tested. HEp2 cells were transfected by electroporation, and BALB/3T3 cells were transfected by the calcium phosphate method, as described in Materials and Methods. Some cells transfected with the reporter plasmid and control plasmids were exposed to the phorbol ester TPA or to recombinant human TNF-α treatments which stimulate NF-κB activity in some cells.

As shown before, 293 cells showed high levels of NF-κB activity after expression of LMP and NFKB1-RelA but not after expression of LMP-NΔ43 (Fig. 5). NF-κB activity was also increased in these cells by exposure to TPA and to recombinant human TNF-α. Thus, stimulation of NF-κB activity by LMP in 293 cells correlated with their response to other treatments that induce NF-κB activity.

HEp2 cells showed small, but significant, increases in NF-κB activity after expression of wild-type LMP and after exposure to TPA (Fig. 5). Transfection of pCMV-NFKB1-RelA stimulated high levels of activity, but exposure to recombinant human TNF-α had no effect. Western blot analysis of lysates from transfected HEp2 cells demonstrated that equivalent amounts of wild-type LMP and LMP-NΔ43 were expressed, both of which at levels that were greater by at least a factor of 10 than those seen in populations of transfected 293 cells (Fig. 5, inset). Thus, wild-type LMP expression induced an increase in NF-κB activity, compared with the activity of LMP-NΔ43. But, the increase observed was small relative to that seen with wild-type LMP in 293 cells or with HEp2 cells expressing NFKB1-RelA.

We tested BALB/3T3 cells under a variety of conditions, none of which showed greater stimulation of NF-κB by wild-type LMP than by LMP-NΔ43. These conditions included culture in medium supplemented with 0.3, 0.5, or 9% CS, periods of serum deprivation, culture in 0.3% CS, which extended from 48 to 60 h, and other methods of transfection (data not shown). Various amounts of pSV_2LMP and pSV_2LMP-NΔ43 plasmid DNAs were also tested, with no effect. At some plasmid concentrations tested, expression of wild-type LMP reduced the activity of 3X-κB-L to a fraction of its basal activity, a pattern that is consistent with the cytotoxic activity of LMP at higher levels of expression (data not shown). Thus, BALB/3T3 cells were tested under conditions that allowed apparently efficient transfection, as judged by the high basal activity of p3X-κB-L, and with amounts of plasmid DNAs that in some cases were sufficiently high to cause LMP-specific cytoxicity, without detection of increases in NF-κB activity that could be attributed to wild-type LMP. Our failure to observe more induction of NF-κB activity by wild-type LMP than by LMP-NΔ43 in BALB/3T3 cells indicates that the transforming function of LMP in these cells does not correlate with changes in the gross levels of NF-κB-mediated transcription detected by the reporter p3X-κB-L.

DISCUSSION

LMP stimulates transcription that is mediated by one or more members of the NF-κB family of transcription factors. LMP increased expression in Jurkat and 293 cells from reporter plasmids that contained κB enhancer elements but not from plasmids that lacked the elements. This finding was similar to those published previously (21, 29). We found also that the NF-κB inhibitor IκB-α reduced LMP stimulation of the activity of the reporter plasmid 3X-κB-L. This inhibition indicated that the majority of the detected stimulation by LMP was mediated by NF-κB.

We demonstrated that the stimulation of high levels of NF-κB-mediated transcription in 293 cells was an attribute of wild-type LMP, because several mutant derivatives of LMP stimulated low or minimal levels of transcription. A comparison of the previously described properties of mutant derivatives of LMP shows that some of the properties are correlated with stimulation of NF-κB activity by LMP (Table 2). Wild-type LMP is associated with the cytoskeleton, forms localized patches in the plasma membrane, and has a short half-life in BALB/3T3 and HEp2 cells (37). Mutant derivatives such as LMP-NΔ43 and LMP-Δ25-132 stimulate minimal NF-κB activity and are not associated with the cytoskeleton at levels that are above the background of detection, nor are they localized in patches in the membrane, turned over rapidly, or transforming or cytotoxic.

The intermediate NF-κB-stimulating activity showed by derivatives of LMP that have truncated carboxy termini, such as LMP-CΔ55 (Table 2), correlated with their intermediate cytoskeletal attachment and turnover in BALB/3T3 and in HEp2 cells (37) but not with their wild-type levels of transforming function in BALB/3T3 cells (4, 37) and cytotoxic activity in HEp2 and other cells (20). LMP stimulated little or no NF-κB activity when expressed in these cell lines. These observations indicate that the stimulation of NF-κB by LMP reflects some of its biochemical properties, although another activity of LMP may be required for its cytotoxicity and its transforming functions. It is possible that NF-κB activity is required but not sufficient for the effects of LMP expression, such that different cell lines exhibit different responses to LMP in a way that is

correlated with the endogenous levels of NF-κB activity. For example, the carboxy terminus of LMP is observed to be required for transforming activity in Rat-1 cells (41) but not in BALB/3T3 cells. If BALB/3T3 cells were to have high levels of constitutive NF-κB function and Rat-1 cells were to have low levels of NF-κB activity, then Rat-1 cells might be transformed only by derivatives of LMP that stimulate maximally NF-κB activity, i.e., LMP derivatives that retain the carboxy terminus.

It is also possible that LMP induces NF-κB/Rel transcription that is correlated with the cytotoxic and transforming activities of LMP but that cannot be detected by the reporter plasmid 3X-κB-L. However, the MHC class I κB elements contained in this reporter plasmid bind multiple NF-κB/Rel heterodimers, including NFKB1-RelA, NFKB1–c-Rel, RelA–c-Rel, NFKB2–c-Rel, and NFKB2-RelA, which are induced in HeLa S3 cells by TNF-α (8).

A consensus SH3-binding domain specified by proline-rich sequences of the cytoplasmic amino terminus of LMP was not required for stimulation of NF-κB. Portions of proteins with appropriately spaced proline residues mediate binding to SH3 domains (13, 47, 57), the *src* homology regions identified in several signal-transducing molecules (reviewed in reference 50). The 24 amino acid residues that compose the cytoplasmic amino terminus of LMP contain up to three SH3-binding motifs, PXXP. When expressed as part of a fusion protein, this region of LMP mediates binding to an SH3-binding partner in solution and in filter-binding assays (10). We found that deletion of the amino acid residues that would be required for SH3-binding activity had no effect on the NF-κB-stimulating function of LMP. This result indicated that any sequence-specific activity of the amino terminus is unlikely to be required for LMP to stimulate NF-κB. Rather, the amino terminus may play a role in NF-κB stimulation that is limited to stabilizing the structure of the adjacent membrane-spanning domain, as has been proposed with respect to the immortalizing activity of LMP (24).

The transmembrane domain of LMP together with a region that lies within the last 55 residues of its carboxy terminus was required for maximal stimulation of NF-κB activity. The low activity of LMP derivatives that lacked portions of the transmembrane domain or that lacked sequences adjacent to membrane-spanning segments, as with LMP-NΔ25 and LMP-CΔ199, indicated that the membrane-spanning segments of LMP are essential for its function. This conclusion has been reached consistently with respect to the other known activities and properties of LMP (4, 20, 37). One hypothesis is that the transmembrane domain binds cellular proteins within the plasma membrane.

The carboxy-terminal region of LMP that is required for maximal activity appears to enhance the function of the transmembrane domain. LMP-Δ25-132, which retains the carboxy terminus of wild-type LMP, is minimally active with respect to its stimulation of NF-κB and its biochemical features, showing that the carboxy terminus alone confers none of the properties that are associated with the membrane-spanning segments. The carboxy-terminal domain may interact with cellular proteins that in turn facilitate the function of the transmembrane domain, or it may interact with other domains of LMP. This carboxy-terminal region is rich in aspartic acid and serine residues but does not contain the major sites of serine and threonine phosphorylation of LMP (40). It contains two potential SH3-binding motifs of the PXXP class, PHLP and PHGP, which could mediate binding events with cellular proteins.

The stimulation by LMP of NF-κB-mediated transcription demonstrates that LMP can influence one signalling pathway of cells. This finding supports the hypothesis that LMP contributes to the establishment of EBV latent infection by inducing proliferative signals in infected cells, although the mechanism of function of LMP remains unknown. The characterization of LMP and its domains that support the stimulation of NF-κB is a useful contribution for the elucidation of one or more biochemical activities intrinsic to LMP. This stimulation occurs rapidly relative to other phenotypes previously associated with LMP and should be an end point from which to track LMP's mode of action.

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