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Epstein-Barr Virus LMP-1 Natural Sequence Variants Differ in Their Potential To Activate Cellular Signaling Pathways

CERI A. FIELDING,[1] KRISTIAN SANDVEJ,[2] ANJA MEHL,[1] PAUL BRENNAN,[1] MATTHEW JONES,[1] AND MARTIN ROWE[1]*

Section of Infection and Immunity, Department of Medicine, University of Wales College of Medicine, Cardiff CF14 4XN, Wales,[1] and Laboratory of Immunopathology, Institute of Pathology, Kommunehospitalet, DK-8000 Aarhus C, Denmark[2]

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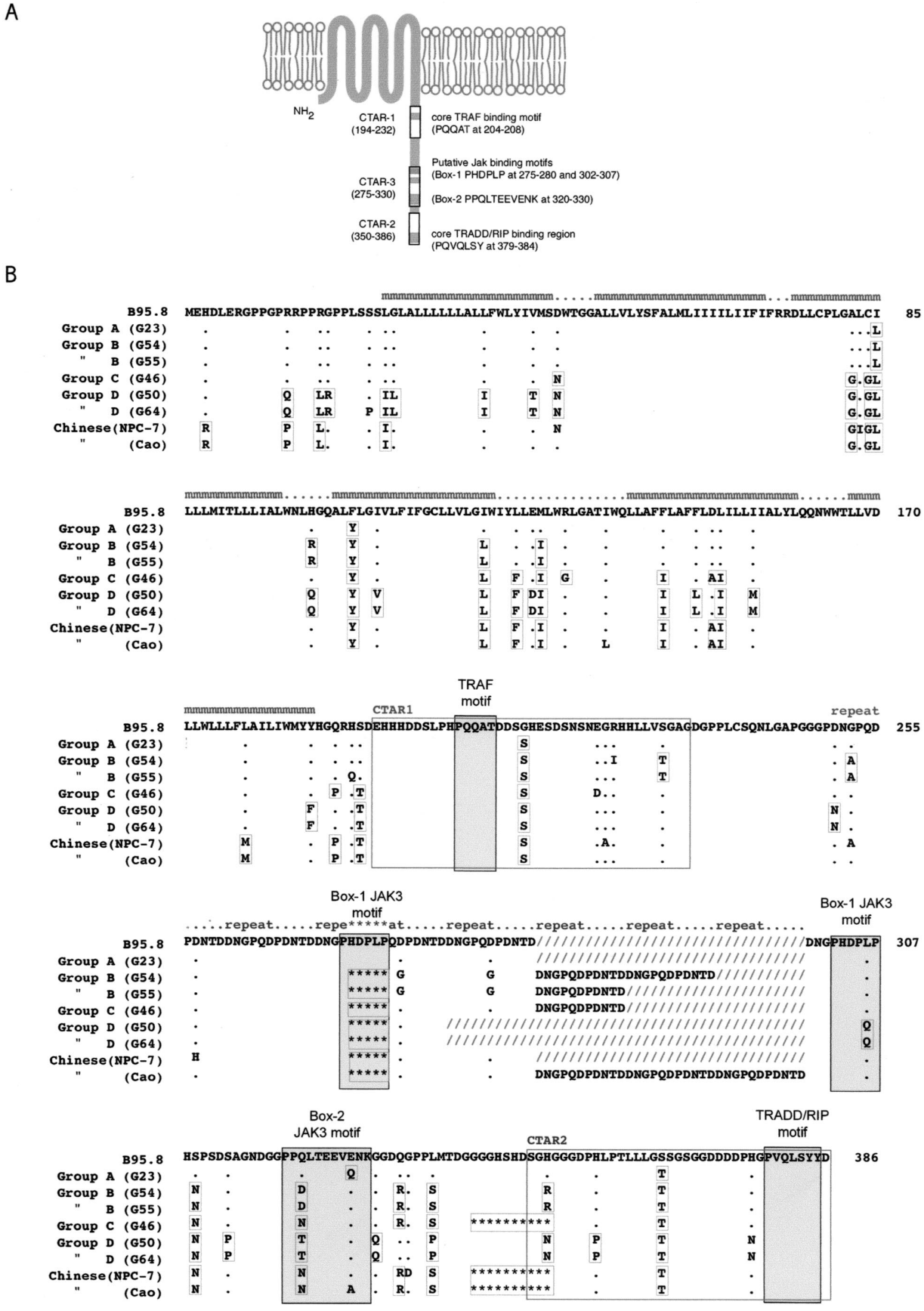
The latent membrane protein 1 (LMP-1) oncogene of Epstein-Barr virus (EBV) is believed to contribute to the development of many EBV-associated tumors, and there is evidence that sequence variation can affect some functions of LMP-1. Most studies have been restricted to the prototype B95.8 LMP-1 gene and genes isolated from EBV of nasopharyngeal carcinoma (NPC) patients. Here, we analyzed the signaling functions of LMP-1 from a panel of nine EBV isolates, including representatives of four defined groups of European LMP-1 variants (groups A to D [K. Sandvej, J. W. Gratama, M. Munch, X. G. Zhou, R. L. Bolhuis, B. S. Andresen, N. Gregersen, and S. Hamilton-Dutoit, Blood 90:323–330, 1997]) and Chinese NPC-derived LMP-1. Chinese and group D variants activated the transcription factor NF-κB two- to threefold more efficiently than B95.8 LMP-1, while Chinese, group B, and group D variants similarly activated activator protein 1 (AP-1) transcription more efficiently than did B95.8 LMP-1. However, there were no amino acid substitutions in the core binding regions for tumor necrosis factor receptor-associated adapter proteins known to mediate NF-κB and AP-1 activation. In contrast, despite sequence variation in the proposed Janus kinase 3 binding region, STAT activation was remarkably constant among the panel of LMP-1 variants. Analysis of the induction of CD54 (intercellular adhesion molecule 1) protein expression by the LMP-1 variants showed differences that did not correlate with either NF-κB or AP-1. Therefore, while the defined sequence variant groups do correlate with LMP-1 function, the results highlight the fact that the relationship between sequence variation and signaling function is extremely complex. It appears unlikely that one particular amino acid substitution or deletion will define a disease-associated variant of LMP-1.

Epstein-Barr virus (EBV) is a potent transforming agent for human B lymphocytes, and infection of resting B cells with EBV in culture regularly gives rise to lymphoblastoid cell lines (LCL) sustaining a largely nonpermissive infection (44). This growth transformation requires the cooperative action of at least five "latent" EBV genes. One of these essential latent genes encodes the latent membrane protein 1, which is oncogenic in various experimental models (3, 74, 77), can enhance cell survival through upregulation of antiapoptotic genes (17, 31, 52, 75), and is essential for continued proliferation of established LCL (47, 55). Furthermore, LMP-1 is expressed in the EBV-associated posttransplant lymphoproliferative disorders (PTLD) in immunocompromised patients, as well as in many EBV-associated malignancies including Hodgkin's disease and nasopharyngeal carcinoma (NPC) (23, 32, 61, 78, 79).

LMP-1 is a transmembrane protein whose essential structural features are illustrated in Fig. 1A. A short cytosolic N terminus of about 23 amino acids interacts with the cytoskeleton and is responsible for turnover of the molecule by the ubiquitin-proteasome degradation pathway (2, 73). The six transmembrane spanning hydrophobic domains serve to induce spontaneous aggregation of LMP-1 molecules, resulting in a constitutively active receptor molecule (27, 29). The large cytoplasmic C terminus of LMP-1 of about 200 amino acids binds various protein adapter molecules utilized by receptors of the tumor necrosis factor (TNF) superfamily (11, 41, 45, 60, 66). These signaling adapter proteins bind to two regions of the LMP-1 molecule termed C-terminal activating regions, CTAR1 and CTAR2, which were originally identified as domains responsible for activation of the NF-κB transcription factors (38, 59). CTAR1, located in the membrane proximal region, has a core PXQXT motif at amino acids 204 to 208 which binds the TNF receptor-associated factors TRAF1, TRAF2, TRAF3, and TRAF5 (11, 15, 66). CTAR2, located at the extreme C terminus of the LMP-1 molecule, binds the TNF receptor-associated death domain protein (TRADD) and the receptor interacting protein (RIP) (19, 39, 41, 45). Recently, a putative CTAR3 domain encompassing amino acids 275 to 330 was reported to bind Janus kinase 3 (JAK-3) (28). Through the spontaneous aggregation of LMP-1 molecules and the association with cytosolic adapter proteins, LMP-1 transduces a number of cell signaling pathways, leading to activation of the transcription factors NF-κB, activator protein 1 (AP-1), and STAT (10, 20, 28, 30, 46).

There has been considerable interest in the possibility that specific sequence changes in LMP-1 may define more oncogenic genotypes. While some molecular epidemiological studies have failed to establish a correlation between LMP-1 sequence and disease association (18, 43, 72), other studies have identified NPC-derived LMP-1 genes as having elevated cell

* Corresponding author. Mailing address: Section of Infection and Immunity, Department of Medicine, 2nd Floor, Tenovus Building, University of Wales College of Medicine, Cardiff CF14 4XN, United Kingdom. Phone: 44 (029) 20 742579. Fax: 44 (029) 20 743868. E-mail: RoweM@cardiff.ac.uk.



transformation potential and decreased immunogenicity (36, 71). Furthermore, expression of LMP-1 variants in NPC correlates with differences in growth pattern and the clinical course of the disease (34, 35). Most attention has focused on LMP-1 variants containing a deletion of 10 amino acids (amino acids 343 to 352), adjacent to CTAR2. This del-LMP-1 variant was originally identified in Chinese NPC tumors (13, 37) and was subsequently detected in European cases of Hodgkin's disease, PTLD, and peripheral T-cell lymphomas (37, 48–50, 70). In vitro experiments suggested that the del-LMP-1 variant has greater oncogenic potential than the prototype B95.8 EBV-derived LMP-1 (13, 37, 54). However, the significance of the association of the del-LMP-1 variant with EBV-related malignancies is unclear, since the incidence of EBV-associated malignant disease in different geographical locations appears to reflect the incidence of the del-LMP-1 variant in the corresponding healthy population (43, 69). Furthermore, while the prototype Chinese del-LMP-1 variant (Cao) and an African NPC-derived del-LMP-1 variant are both able to induce high levels of NF-κB activation, this property does not map to the 10-amino-acid deletion itself but rather to other associated sequence variations that are not clearly defined (8, 42, 58).

In the present study, because of the difficulty in ascribing an altered LMP-1 phenotype to single amino acid substitutions, it was decided to analyze the functions of a larger panel of naturally occurring LMP-1 genes carefully selected from defined groups of sequence variants. Sequence analysis of the LMP-1 gene and promoter in 62 EBV isolates from European individuals has allowed the definition of four main groups of LMP-1 variants, designated groups A, B, C, and D (69). Each of these four groups is characterized by a specific pattern of amino acid mutations relative to B95.8 LMP-1 (Fig. 1B; Table 1). Only in group C LMP-1 variants is the 10-amino-acid sequence at residues 343 to 352 deleted. Interestingly, none of the group A through D variants or the Chinese del-LMP-1 variants shows mutations in the core TRAF/TRADD/RIP binding sequences in CTAR1 and CTAR2. However, there are numerous mutations in the putative JAK-3-binding Box-1 and Box-2 motifs in CTAR3 (see Fig. 1).

The main question addressed by the present study is whether the natural sequence variation in LMP-1 correlates with the ability to activate the transcription factors that mediate the various biological functions of this oncogene. Previous studies have indicated significant differences between the B95.8 LMP-1 and NPC-derived LMP-1 variants with respect to activation of NF-κB (42, 58). However, there is still a need for a more systematic analysis of a panel of well-defined LMP-1 variants, not only for the ability to activate NF-κB but also for the other transcription factors activated by LMP-1. We therefore compared nine different LMP-1 genes, including the prototype B95.8 LMP-1 and Cao del-LMP-1 variants and representatives of each of the defined groups A, B, C, and D. Activation of the NF-κB, AP-1, and STAT transcription factors was measured by cotransfecting expression plasmids for each of the cloned LMP-1 genes with luciferase reporter plasmids regulated by response elements for these transcription factors. We also investigated the abilities of the cloned LMP-1 variants to induce expression of intercellular adhesion molecule 1 (ICAM-1, also known as CD54) in lymphocytes, since regulation of this cellular gene by LMP-1 has recently been shown to be dependent on new, as yet unidentified signaling events (56). The results of the present study show that natural sequence variation does indeed affect LMP-1 signaling potential.

MATERIALS AND METHODS

Cell lines. Jurkat is an EBV-negative T-cell lymphoma-derived cell line (9). Eli-BL is an EBV-positive B-cell Burkitt's lymphoma, displaying a latency I type of infection, in which EBNA-1 is expressed but the other EBNAs and LMP-1 are not (65). DG75 is an EBV-negative Burkitt's lymphoma cell line (7). CR+B95.8 is an LCL which was established by in vitro infection of normal B cells from an EBV-seronegative donor with B95.8 EBV (62). A number of other EBV-transformed LCLs (G23, G54, G55, G46, G50, and G64) were established by spontaneous outgrowth from cultures of peripheral blood lymphocytes from EBV-positive European individuals (69); these LCLs were kindly provided by Jan Willem Gratama, Rotterdam, The Netherlands. The NPC-7 line is a spontaneous LCL established from the blood of a southern Chinese patient with NPC (57) and was kindly provided by Alan Rickinson, Birmingham, United Kingdom. Cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics (200 U of penicillin/ml and 200 mg of streptomycin/ml) and were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Plasmids. The mammalian expression vector pSG5-neo was produced by insertion of the neomycin resistance cassette from pSV2-neo (Clontech) into the unique *Xba*I site of pSG5 as a blunt-ended fragment. The green fluorescent protein (GFP) expression vector pEGFP-N1 was purchased from Clontech. The 3Enh.κB-ConALuc reporter (3Enh-Luc) has been described previously (1) and contains three tandem repeats of the NF-κB binding sites from the immunoglobulin (Ig) κ promoter upstream of a minimal conalbumin promoter. The pAP1-Luc reporter contains seven tandem copies of an AP-1 binding element fused to a TATA-like promoter region from the herpes simplex virus thymidine kinase promoter (Clontech). The GRR(5)-Luc reporter contains five tandem copies of the FcγR1 gamma interferon-activated sequence upstream of a minimal thymidine kinase promoter in the pBL-Luc vector (6) .

Cloning and sequencing of LMP-1 natural sequence variants. DNA was extracted from washed LCL pellets by lysing in PCR-lysate buffer (10 mM Tris-Cl [pH 9], 1 mM EDTA, 0.5% Nonidet P-40, 0.5% Tween 20, 200 μg of proteinase K/ml) at a concentration of 10^7 cells/ml. The lysate was incubated for at least 3 h at 55°C, and the proteinase K was heat inactivated at 95°C for 15 min. Phenol-chloroform extraction was performed, followed by ethanol precipitation. For the Cao LMP-1 gene, no LCL was available; the source of DNA for this gene was the J124-Cao construct obtained from L.-F. Hu and coworkers (37). The LMP-1 genes were PCR amplified from the DNA using a common antisense primer

FIG. 1. Amino acid sequences of LMP-1 natural sequence variants. (A) Schematic showing some features of the structure of LMP-1 in the plasma membrane. The boxed areas on the C terminus indicate the positions of signaling domains. (B) Alignment of the amino acid sequences of the nine LMP-1 genes analyzed in this study. The complete sequence of B95.8 is shown in single-letter amino acid code. Only differences from the B95.8 sequence are indicated for the other eight LMP-1 sequences. Amino acid substitutions that are shared by all members of a defined group of variants are boxed. Unboxed amino acid substitutions are those that are present in the particular isolate studied but are not characteristic of all members of its group. Asterisks indicate deletions. Transmembrane domain residues are indicated by "mmm" above the B95.8 sequence. Eleven-amino-acid repeats are indicated by "repeat" above the B95.8 sequence, and their absence in the aligned sequence is indicated by a series of slashes. The CTAR1 and CTAR2 domains are indicated by open boxes enclosing the sequences. Core motifs known to be essential for binding of TRAF, TRADD, or RIP, and motifs reported to be potential JAK-3 binding sites, are indicated by shaded boxes enclosing those sequences. Note that the amino acid sequence of Cao LMP-1 differs from the previously published sequence (see the text). The corrected sequence has been entered in the GenBank database (accession number AF30442).

TABLE 1. Defining mutations of the group A, B, C, and D and Chinese NPC variants

Location[a]	Codon	Amino acid(s)[b] in:					
		Reference sequence (B95.8)	Group A	Group B	Group C	Group D	Chinese NPC
N-term	3	H	—	—	—	—	R
	13	R	—	—	—	Q	P
	17	R	—	—	—	L	L
	18	G	—	—	—	R	—
	25	L	—	—	—	I	I
Tm	26	G	—	—	—	L	—
	37	L	—	—	—	I	—
	43	V	—	—	—	T	—
Loop out	46	D	—	—	N	N	—
Tm	82	A	—	—	G	G	G
	84	C	—	—	G	G	G
	85	I	L	L	L	L	L
Loop out	101	H	—	R	—	Q	—
Tm	106	F	Y	Y	Y	Y	Y
	109	I	—	—	—	V	—
	122	I	—	L	L	L	L
Loop in	126	L	—	—	F	F	F
	128	E	—	—	—	D	—
	129	M	—	I	I	I	I
	132	R	—	—	G	—	—
Tm	144	F	—	—	I	I	I
	148	F	—	—	—	L	—
	150	D	—	—	A	—	A
	151	L	—	—	I	I	I
	155	I	—	—	—	M	—
	178	L	—	—	—	—	M
C-term	186	Y	—	—	—	F	—
	189	Q	—	—	P	—	P
	192	S	—	—	T	T	T
	212	G	S	S	S	S	S
	229	S	—	T	—	—	—
	250–298	Repeats	Variable	Variable	Variable	Variable	Variable
	306	L	—	—	—	Q	—
	309	S	—	N	N	N	N
	313	S	—	—	—	P	—
	322	Q	—	D	N	T	N
	328	E	Q	—	—	—	—
	331	G	—	—	—	Q	—
	334	Q	—	R	R	—	R
	338	L	—	S	S	P	S
	343–352	GGGHSHDSGH	—	—	Deletion	—	Deletion
	352	H	—	R	—	N	—
	358	H	—	—	—	P	—
	366	S	T	T	T	T	T
	377	H	—	—	—	N[c]	—

[a] N-term, amino terminus; Tm, transmembrane; Loop in, transmembrane loop exposed to cytoplasm; Loop out, transmembrane loop exposed to outside of cell; C-term, carboxy terminus. Substitutions in the N-term, Loop in, and C-term rows were localized to the cytoplasm.

[b] —, no change from the B95.8 amino acid. Substitutions relative to the B95.8 amino acid are indicated by the single-letter code.

[c] The H377N substitution is present in about 50% of group D variants and was present in both of the group D variants (G50 and G64) analyzed in this study.

(5′-ACGCGTTTCTGCGGATCAGTCG-3′) but different sense primers according to the LMP-1 isolate. For PCR amplification from B95.8, G23, G54, G55, and G46, the sense primer was 5′-CTCCTGACACACTGCCCTGAG-3′. For amplification from G50 and G54, the sense primer was 5′-CTCCTGCCACACTACC CTGAG-3′. For amplification from NPC-7 and Cao, the sense primer was 5′-CTCCTGCCACACTACCCTGAC-3′.

The purified PCR products were incorporated into the pCR2.1 vector (T-A Topo Cloning kit; Invitrogen). Each LMP-1 gene was excised from the pCR2.1 vector as an *Eco*RI fragment and ligated into the *Eco*RI site of SG5-neo. The exception was the G23 LMP-1 gene, which was found to have an *Eco*RI restriction site in the first intron; the G23 LMP-1 gene was therefore PCR amplified from the pCR2.1 vector with primers incorporating additional 5′ *Bam*HI sites and was ligated into the *Bam*HI site of SG5-neo. The expected number of repeats in each cloned gene was confirmed using a diagnostic PCR adapted from the work of Khanim et al. (43). The cloned LMP-1 genes were then sequenced with the ABI Big Dye sequencing kit (Perkin-Elmer Applied Biosystems) with

various primers for the SG5 vector and the LMP-1 gene to give complete overlapping nucleotide sequences of the entire LMP-1 coding region. The B95.8 and Cao LMP-1 genes were both recloned for the present study; the results obtained in functional assays with the newly cloned vectors reproduced our previously published results with these LMP-1 variants, thus ruling out possible cloning artifacts as an explanation for the different phenotypes.

Gene transfection and luciferase reporter assay. Up to 10^7 cells in 0.5 ml of RPMI medium buffered with 100 mM HEPES (pH 7.2) were transfected by electroporation using a Bio-Rad Genepulser II electroporator set to 280 V and 950 μF for Jurkat and Eli-BL cells, or 270 V and 950 μF for DG75 cells. The cells were then seeded in 4 ml of fresh growth medium and cultured under normal conditions. Transfection efficiency was typically 20 to 30% for the Jurkat cell line, 5 to 10% for the Eli-BL cell line, and 40 to 50% for the DG75 cell line. Luciferase activity from the reporter constructs was measured at 24 h posttransfection, exactly as described previously (56).

Assay for cell surface CD54 (ICAM-I) by flow cytometry. The induction of cell surface CD54 protein in the transfected cell population was assayed by immunofluorescence staining of viable cells, followed by flow cytometry using a Becton Dickinson FACScalibur analyzer as described previously (56). Briefly, cells were harvested 48 h after transfection and stained with a phycoerythrin (PE)-conjugated monoclonal antibody to human CD54. The transfected cell population was marked by the expression of cotransfected EGFP-N1 plasmid, and this population was gated for analysis of CD54 staining.

Detection of LMP-1 by immunoblotting. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and immunoblotting were performed as described previously (56). Briefly, cells were lysed in gel sample buffer (50 mM Tris [pH 6.8], 100 mM dithiothreitol, 2% SDS, 10% glycerol, 0.05% bromophenol blue), sonicated, and boiled. Solubilized proteins equivalent to 2×10^5 cells were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes for immunoblotting. LMP-1 was detected by first incubating the membranes for 1 h with 1 μg of CS.1-4/ml (63) and then incubating for 1 h with a 1/10,000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG (Bio-Rad catalog no. 170-6461). Specific antibody-protein complexes were detected using the CDP-Star (Tropix Inc.) chemiluminescence reagent.

Detection of LMP-1 by immunofluorescence. Air-dried smears of transfected DG75 cells were fixed with acetone-methanol (1:1) and then stained with 10 μg of a mouse monoclonal antibody to LMP-1 (CS.1)/ml (63), followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (F2012; Sigma) as described previously (64). The stained cells were visualized on a Leica DM LB microscope.

Nucleotide sequence accession number. The corrected amino acid sequence of Cao LMP-1 has been entered in the GenBank database (accession number AF30442).

RESULTS

Cloning and sequencing of LMP-1 natural sequence variants. As a first step in determining whether sequence variation affected LMP-1 signaling potential, a panel of nine LMP-1 genes was cloned into the SG5-neo expression vector. The amino acid sequences of these nine LMP-1 genes are shown in Fig. 1B. As references, the B95.8 LMP-1 and Cao LMP-1 genes were also recloned, sequenced, and included in this panel. It should be noted that we have sequenced both the recloned Cao LMP-1 gene and the original gene obtained from L.-F. Hu and coworkers and have found discrepancies with the published sequence (37). The corrected Cao LMP-1 sequence (GenBank accession number AF30442) is shown in Fig. 1B.

Six of the other LMP-1 genes were cloned from spontaneous LCL derived from healthy European individuals and were selected in the knowledge that the LMP-1 genes for these individuals had been previously sequenced and classified into one of four defined groups, A through D (69). The group A, B, C, and D LMP-1 genes are defined on the basis of consistent nucleotide mutations, which occur in every member of a defined group. Those defining mutations which lead to amino acid substitutions are listed in Table 1 and indicated in the sequences shown in Fig. 1B. Excluding the variable-repeat region (residues 250 to 298 in B95.8 LMP-1), group A variants show 5 amino acid substitutions with respect to the B95.8 LMP-1 sequence, group B variants show 13 substitutions, group C variants show 20 substitutions, and group D variants show 34 substitutions (Fig. 1B). In addition to these consistent defining mutations, other occasional mutations were observed in different members of a group. Thus, we analyzed two representatives of the group B variants, each having a single, but different, additional amino acid substitution: R233I in the G54 LMP-1 and H191Q in the G55 LMP-1 (Fig. 1B). Similarly, we analyzed two members of the group D variants; G50 and G64 share one additional substitution, H377N, and G64 also has a second substitution, S23P, in addition to those present in all group D variants. In this context, it should be noted that 4 of the 5 defining changes in group A variants (I85L, F106Y, G212S, and S366T, but not E328Q) are also found in the group B, C, and D variants and in the two Chinese isolates (Table 1). Furthermore, like B95.8, group A variants contain a 5-amino-acid insert in the repeat region (residues 275 to 280) which is not observed in any other variant group (Fig. 1B). Together, these observations suggest that B95.8 is an example of a group A variant.

The NPC-7 LMP-1 gene was obtained from a southern Chinese NPC patient (57). It shows only 7 amino acid substitutions relative to the prototype Cao LMP-1, and these are mostly conservative changes (Fig. 1B). Together, NPC-7 and Cao share 23 substitutions relative to B95.8 (Table 1). Both NPC-7 and Cao correspond to the "Chinese-1" subgroup of LMP-1 variants identified by Edwards et al. (18). The 10-amino-acid deletion (corresponding to residues 343 to 352 of B95.8) is shared by NPC-7 and Cao, as well as by European group C variants, represented by G46 LMP-1. Interestingly, while group C variants most closely resemble the Chinese variants in the transmembrane and C-terminal regions, the N-terminal regions of these two groups are quite distinct. The N-terminal regions of the Chinese variants show the greatest similarity to group D European variants.

The number of 33-bp (11-amino-acid) repeats varies independently of the defined groups. Thus, Fig. 2a shows the sizes of PCR-amplified DNA fragments obtained, using primers flanking the repeat sequences, from each of the nine LMP-1 genes in the SG5-neo vector. This experiment shows, for example, that the two group B isolates, G54 and G55, have 6 and 5 repeats, respectively. Since the panel of representative LMP-1 variant genes had been cloned into the SG5-neo vector, which contains a heterologous simian virus 40 (SV40) promoter, it was necessary to confirm expression of the correct LMP-1 proteins.

Expression of LMP-1 natural sequence variants. SV40 promoter-driven expression of the LMP-1 genes from the SG5-neo vector was first confirmed by immunoblot analysis of proteins extracted from Jurkat cells transfected with the respective plasmids. A representative blot, probed with monoclonal antibodies CS.1-4, is shown in Fig. 2B. This experiment illustrates the expected variation in protein size, which is due to the combined effects of the variable number of 11-amino-acid repeats, the presence or absence of the 5 amino acids inserted in the 4th perfect repeat, and the presence or absence of the 10-amino-acid deletion within CTAR2 (see Fig. 1B). This Western blot also suggests that both group B variants, G54 and

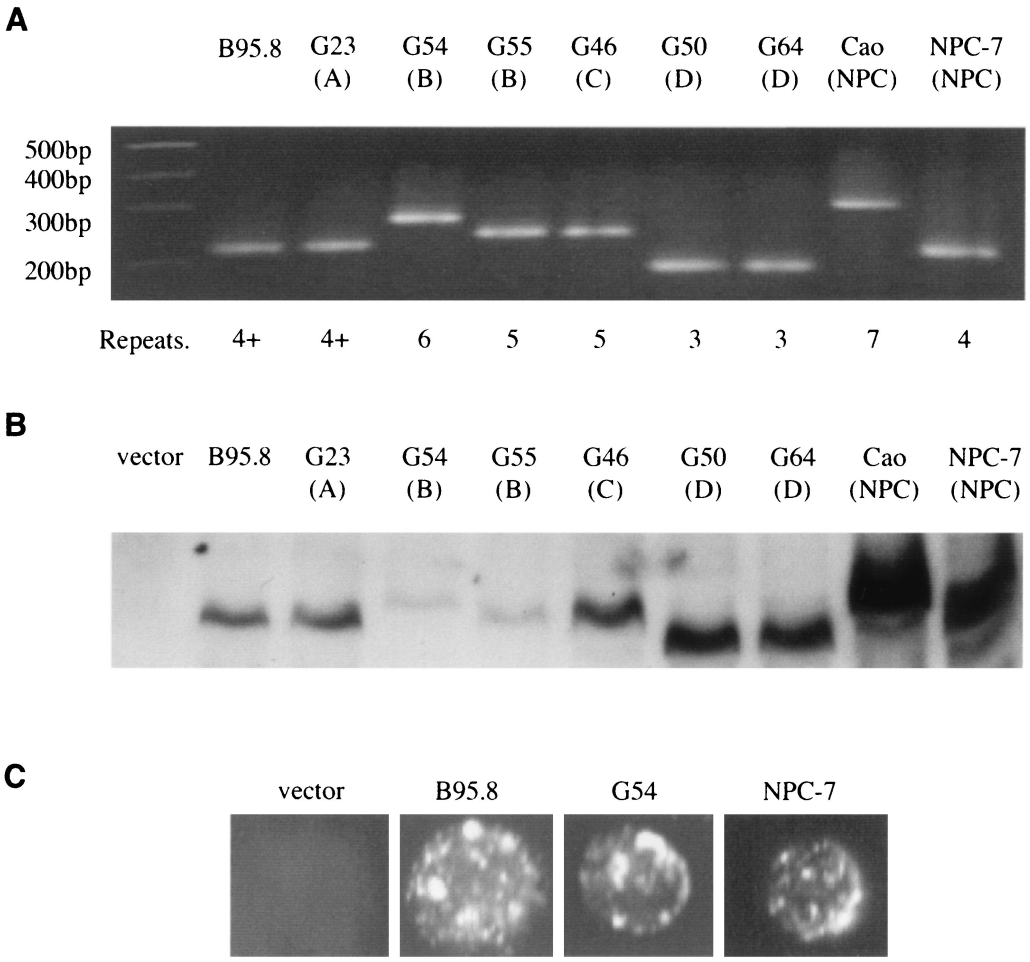


FIG. 2. Characterization and expression of cloned LMP-1 variants. (A) Agarose gel showing differences in the number of 11-amino-acid (33-bp) repeats detected by PCR amplification. PCR products were resolved on a 3% agarose gel with a DNA ladder, stained in an ethidium bromide solution, and visualized with UV light. The sizes of DNA ladder fragments are given in base pairs, and the number of repeats identified by the size of the PCR product is shown beneath each lane. The B95.8 and G23 LMP-1 genes contain 4+ repeats, which corresponds to four perfect 33-bp (11-amino-acid) repeats and the 15-bp (5-amino-acid) sequence that is deleted in all other LMP-1 variants used. (B) Immunoblot showing expression of the LMP-1 variants from the pSG5-neo vector in transiently transfected Jurkat cells. Vector (pSG5-neo)-transfected Jurkat cells were used as a negative control. Approximately 2×10^5 cells were separated on a 10% polyacrylamide gel and transferred to a PVDF membrane. LMP-1 expression was detected by probing blots with the CS.1-to-CS.4 pool of monoclonal antibodies to LMP-1, followed by an alkaline phosphatase-based chemiluminescence detection protocol. (C) Immunofluorescence staining of LMP-1 in transiently transfected DG75 cells. DG75 cells were transfected with 5 μg of LMP-1 expression vector or empty vector. Cell smears were stained with monoclonal antibody CS.1 to the C terminus of LMP-1 and an FITC-conjugated goat anti-mouse IgG as a secondary antibody. Images of a single cell transfected with either empty vector, B95.8 LMP-1, G54 LMP-1, or NPC-7 LMP-1 are shown.

G55, may have reduced expression relative to other variants. However, this should be interpreted with caution, since the LMP-1 antibodies that are reactive in Western blots are specific for peptide sequences in or around the repeat region of the cytosolic C terminus and may be particularly affected by sequence variation.

Antibody CS.1, which recognizes a peptide epitope at the conserved extreme C terminus (unpublished data), is less likely to be affected by sequence variation. By itself, this antibody does not work well in immunoblotting (63), but it does react well with LMP-1 in immunofluorescence staining of fixed cell smears (Fig. 2C). Immunofluorescence staining of G54 (Fig. 2C) and G55 (data not shown) with CS.1 did not indicate marked differences in levels of expression. Furthermore, the immunofluorescence staining revealed characteristic concentration or "patching" of LMP-1 expression in DG75 cells transfected with each of the nine variant LMP-1 expression plasmids (Fig. 2C; also data not shown), indicating that none of the sequence variations grossly affected cellular localization. Although the levels of expression could have been quantified more accurately by inserting antibody tag sequences into each of the cloned variants, we were reluctant to do this because of uncertainty about the subtle effects that the tags may have on LMP-1 function. Instead, in the following experiments, analyses of transcriptional activation of reporters were carried out by careful titration of each variant LMP-1 plasmid in each type of reporter assay to give dose-response curves from which optimal amounts of LMP-1 plasmids for each assay could be determined.

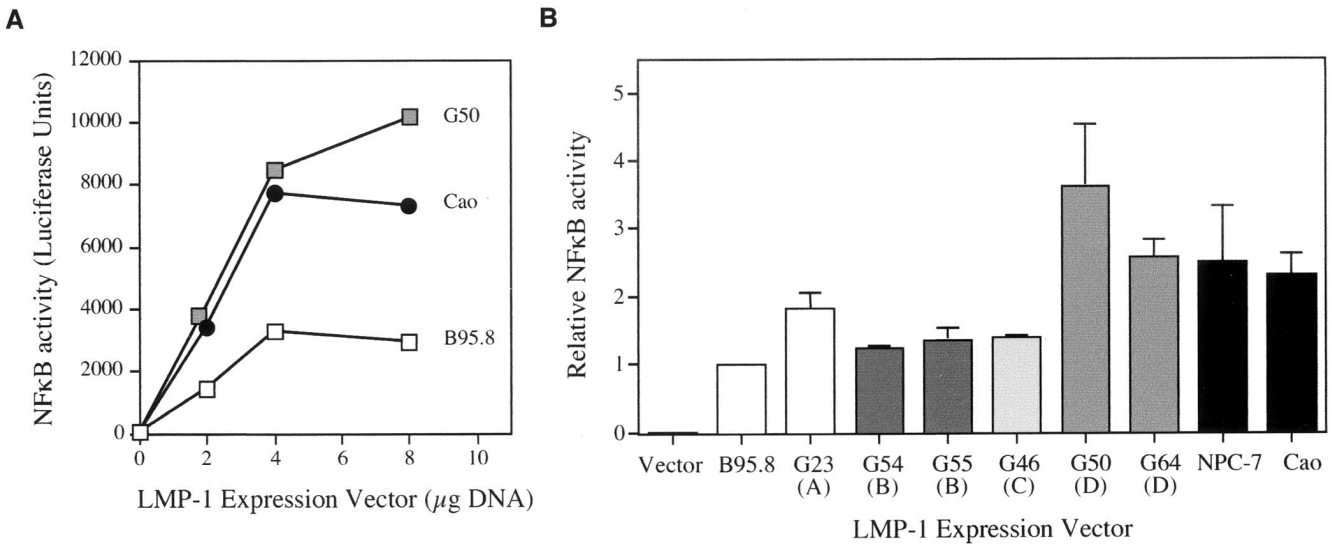


FIG. 3. Activation of NF-κB-regulated transcription by the panel of LMP-1 genes. (A) Dose-response experiment, with up to 8 μg of LMP-1 expression vectors cotransfected with 3 μg of the 3Enh-Luc reporter in the Jurkat cell line. The total amount of DNA per transfection was kept constant by addition of an appropriate amount of empty vector (SG5-neo). Luciferase activity was measured after 24 h. Results of one representative experiment, in which the B95.8, G50 (group D), and Cao (Chinese NPC) LMP-1 were transfected in parallel, are shown. (B) Summary of the relative NF-κB activity induced by each of nine variant LMP-1 genes. Results are means from at least three experiments, and the maximum activation of NF-κB by each variant gene is shown relative to the maximum activation observed with B95.8 LMP-1 in each experiment. Error bars indicate 1 standard deviation from the mean.

NF-κB activation by LMP-1 natural sequence variants. NF-κB has been shown to be involved in the LMP-1-mediated regulation of many cellular genes, e.g., upregulation of A20 (52), bcl-2 (24), c-IAP2 (33), interleukin-6 (IL-6) (21), IL-8 (20), TRAF1 (16), CD40 (16), and CD54 (56) and downregulation of CD99 (53). In order to investigate the ability of our panel of LMP-1 variants to activate NF-κB, the LMP-1 expression plasmids were cotransfected with the 3Enh-Luc reporter into the Jurkat cell line. Luciferase reporter activity was measured at 24 h postinfection. A dose-response curve for each LMP-1 variant was obtained by transfection of a variable amount of LMP-1 expression vector with empty vector (to a total amount of 8 μg) together with 3 μg of 3Enh-Luc reporter. Figure 3A shows results of a representative dose-response experiment for three LMP-1 variants: B95.8 (group A), G50 (group D), and Cao (Chinese NPC) LMP-1. As shown in Fig. 3A, maximum activation of NF-κB was typically obtained with 4 to 8 μg of LMP-1 expression vector. Figure 3A also shows that the G50 and Cao LMP-1 genes activated NF-κB about 2.5- to 3-fold more efficiently than the B95.8 LMP-1 gene. Replicate titrations were performed for each of the variant LMP-1 genes. In order to compare the results of several batches of experiments performed on separate occasions, the B95.8 gene was included in every experiment as a reference, and the maximum NF-κB-mediated luciferase activity was expressed relative to this reference. Figure 3B summarizes the results obtained with all the LMP-1 genes studied.

Figure 3B shows that all the LMP-1 genes were able to activate NF-κB but that they fell into two sets with regard to the degree of activation. The group D and Chinese NPC variants typically gave maximum levels of NF-κB activation at least twofold higher than those of the group A, B, or C variants. The most striking similarity between the amino acid sequences of group D LMP-1 and the Chinese NPC LMP-1 lies in the N terminus, where these two variant groups share substitutions at 3 residues (R13Q/P, R17L, and L25I) relative to all other variant groups. These data therefore suggest that sequence variation in the cytosolic N terminus can influence the efficiency with which LMP-1 activates NF-κB. Next, we questioned whether the same pattern of enhanced signaling would be observed for other transcription factors activated by LMP-1.

AP-1 activation by LMP-1 natural sequence variants. Many of the cellular genes regulated by LMP-1 contain enhancer elements for AP-1 transcription factors, although the direct involvement of LMP-1-activated AP-1 in transcriptional regulation has not been well studied. Nevertheless, it is clear that LMP-1 can activate AP-1 through a c-Jun N-terminal kinase (JNK) pathway, via the TRADD/RIP binding element in CTAR2 of the LMP-1 (22, 46). AP-1 appears to be important for the production of IL-8 induced by LMP-1 (20) and may contribute to the development of Hodgkin's disease by protecting cells from apoptosis (51). NF-κB can be activated via either CTAR 1 or CTAR2, whereas AP-1 can be activated only via the CTAR2 region of LMP-1. Therefore, it was of interest to see whether there were differences in the efficiency with which variants of LMP-1 activated AP-1 and whether the variants segregated in the same way as they did with regard to the efficiency of NF-κB activation.

To investigate the ability of our panel of LMP-1 variants to activate AP-1, the LMP-1 expression plasmids were cotransfected with the AP1-Luc reporter into the Jurkat cell line, and luciferase activity was measured at 24 h. As with the NF-κB reporter experiments, a dose-response curve for each LMP-1 variant was obtained by transfection of a variable amount of LMP-1 expression vector with empty vector (to a final amount of 8 μg) and 3 μg of AP-1-Luc reporter. Figure 4A shows results of a representative experiment for three LMP-1 genes: B95.8 (group A), G23 (group A), and G54 (group B). The

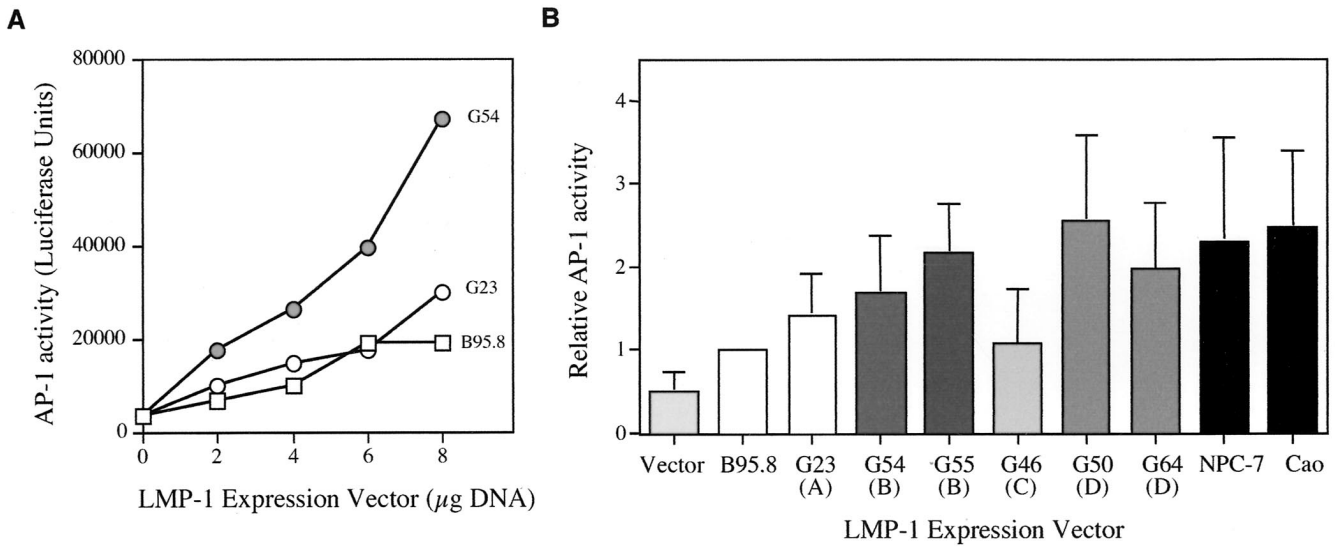


FIG. 4. Activation of AP-1-regulated transcription by the panel of LMP-1 genes. (A) Dose-response curves, with up to 8 μg of LMP-1 expression vectors cotransfected with 3 μg of the AP-1-Luc reporter in the Jurkat cell line. The total amount of DNA per transfection was kept constant by addition of an appropriate amount of empty vector (SG5-neo). Luciferase activity was measured after 24 h. Results of one representative experiment, in which B95.8, G23 (group A), and G54 (group B) LMP-1 genes were transfected in parallel samples, are shown. (B) Relative activation of AP-1 by the LMP-1 variants. Mean AP-1 activities induced by LMP-1 sequence variants are expressed relative to that of B95.8 LMP-1 from at least five replicate transfections with 8 μg of LMP-1 expression vector. Error bars indicate 1 standard deviation from the mean.

B95.8 and G23 genes followed very similar dose-response curves, with relatively poor induction of AP-1, whereas the G54 gene consistently gave higher AP-1 activity than the group A variant at every dose. In contrast to the dose-response experiments for NF-κB activation (Fig. 3A), the dose responses obtained with the AP-1 reporter (Fig. 4A) did not reproducibly peak within the concentration range tested. Since reproducible differences between the LMP-1 variants were obtained at 8 μg of LMP-1 plasmid DNA, replicate experiments were performed with the whole series of LMP-1 expression vectors at this single concentration.

Figure 4B shows the relative AP-1 activation for each LMP-1 variant compared to B95.8 LMP-1. Even though these data are means from at least five experiments, the low fold activation of AP-1 above basal activity by B95.8 LMP-1 caused the standard deviations to be relatively large (compare Fig. 4B with Fig. 3B and 5B). Nevertheless, a reproducible pattern of results was obtained. B95.8, group A, and group C LMP-1 variants showed relatively poor induction of AP-1 activity, while group B, group D, and Chinese NPC variants typically induced AP-1 activation to two- to threefold above B95.8 LMP-1 activity. The AP-1 results therefore differed from the NF-κB results shown in Fig. 3B, with the group B variants falling into the "low" NF-κB-activating set but the "high" AP-1-activating set. There are no obvious amino acid sequence changes common to group B, group D, and Chinese NPC variants which could be responsible for the increase in AP-1 activation. In particular, any changes within CTAR2 that are common to these three groups were also present in group C LMP-1, which activates AP-1 poorly (Fig. 1B and 3). The increased AP-1 signaling observed with the group D and NPC variants could be due to the same changes that caused the increased NF-κB signaling, in which case different amino acid changes may be responsible for the increased activity of group B variants.

The results with NF-κB and AP-1 in Fig. 3 and 4 illustrate two points: (i) that sequence variation can significantly affect the efficiency of activation of cellular transcription and (ii) the difficulty in predicting the efficiency of transcription factor activation from what we already know about the functional domains of LMP-1 and the signal transduction mechanisms. We therefore decided to extend the analysis to a third family of transcription factors activated by LMP-1, the STATs, since the reported effector domains on LMP-1, the putative JAK-3-binding motifs within CTAR3 (28), are subject to substantial variation in amino acid sequence among different groups of LMP-1 genes (Fig. 1).

STAT activation by LMP-1 natural sequence variants. Members of the STAT family of transcription factors are constitutively activated in LCLs, NPC, and Hodgkin's disease (12, 76). LMP-1 has the ability to activate STATs (10, 28), and many cellular genes induced by LMP-1 contain STAT-responsive regulatory elements. We tested the ability of LMP-1 natural variants to activate STATs, using the GRR(5)-Luc luciferase reporter, which contains five copies of a gamma interferon-activating element. LMP-1 has previously been shown to activate this reporter (10), although which particular STATs are involved in this activation has not yet been determined.

Figure 5A shows results of a representative experiment in which variable amounts of LMP-1 expression vector with empty vector (to a final amount of 6 μg) were cotransfected with 10 μg of GRR(5)-Luc reporter into the Eli-BL cell line. This experiment incorporated dose responses for four LMP-1 genes: B95.8 (group A), G54 (group B), NPC-7, and Cao (Chinese NPC). Similar results were obtained with all four

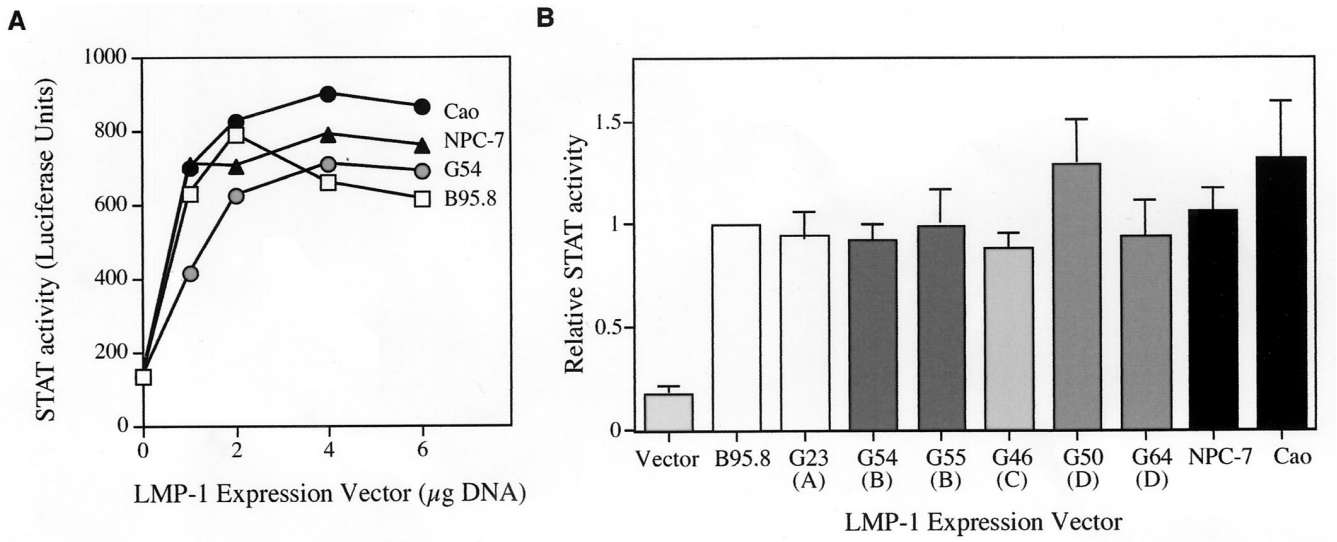


FIG. 5. Activation of STAT-regulated transcription by the panel of LMP-1 genes. (A) Dose-response curves with up to 6 μg of LMP-1 expression vectors cotransfected with 10 μg of the GRR(5)-Luc reporter in the Eli-BL cell line. The total amount of DNA per transfection was kept constant by addition of an appropriate amount of empty vector (SG5-neo). Luciferase activity was measured after 24 h. Results of one representative experiment, in which the B95.8, G54 (group B), and Cao and NPC-7 (Chinese NPC) LMP-1 genes were transfected in parallel samples, are shown. (B) Relative activation of STAT by the LMP-1 variants. Results are means from at least five experiments, and the maximum activation of STAT by each variant gene is shown relative to the maximum activation observed with B95.8 LMP-1 in each experiment. Error bars indicate 1 standard deviation from the mean.

LMP-1 genes, and maximum STAT activation was observed at 2 to 4 μg of LMP-1 expression vector. Figure 5B summarizes the results of replicate experiments with all the variant LMP-1 genes, comparing the maximum STAT activation. In contrast to the results obtained with NF-κB and AP-1 (Fig. 3B and 4B), Fig. 5B shows that the level of STAT activation was remarkably consistent among all LMP-1 variants. G50 LMP-1 and Cao LMP-1 induced slightly higher STAT activation than the other variants, but this did not reach statistical significance. The consistency of the STAT results raises the possibility that the activation of STAT-regulated transcription by LMP-1 is not primarily determined by binding of JAK-3 to the Box-1 and Box-2 motifs in CTAR3.

CD54 upregulation by LMP-1 natural sequence variants. Previous studies on the upregulation of cell surface CD54 (ICAM-I) protein by LMP-1 have revealed additional, as yet unidentified LMP-1-induced signaling events other than those involving NF-κB, AP-1, and STAT (56). This downstream biological readout for LMP-1 was therefore included in the present analysis in order to detect other possible differences in LMP-1 function due to natural sequence variation. The panel of LMP-1 sequence variants was examined for the ability to upregulate CD54 expression on Jurkat cells. Cells were cotransfected with 8 μg of LMP-1 plasmids and 2 μg of GFP expression plasmid, and the expression of CD54 on transfected cells was determined at 48 h posttransfection by flow cytometry using PE-conjugated anti-CD54 antibodies. Gating for GFP fluorescence allowed selective analysis of the transfected subpopulation.

Figure 6 summarizes the results of three separate experiments, where the induction of CD54 for each LMP-1 variant was expressed relative to the induction obtained with B95.8 LMP-1. The group A and Chinese NPC LMP-1 variants showed indistinguishable levels of induced CD54, while the group B and group C LMP-1 variants showed a small but reproducible impairment (20 to 30%) of induction relative to B95.8. The group B LMP-1 variants (G54 and G55) contain a number of unique amino acid substitutions that could contribute to the small reduction in CD54 induction (e.g., H352R), but none that is shared with the group C variant (Fig. 1B). With regard to the group C amino acid substitutions, one (R123G) is of particular interest, since it was shown previously

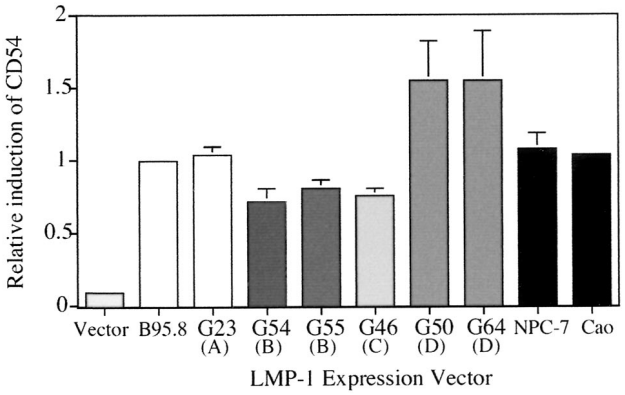


FIG. 6. Relative induction of CD54 (ICAM-1) by LMP-1 variants. Levels of CD54 protein induced in Jurkat cells by the LMP-1 sequence variants were measured at 48 h posttransfection by immunofluorescent staining with PE-conjugated anti-CD54 antibodies and analyzed by flow cytometry. Transfected cells were identified and gated by expression of cotransfected GFP marker plasmid, and these cells were selectively analyzed for CD54 protein expression. Results for each variant were expressed relative to the induction of CD54-positive cells observed with B95.8 LMP-1. The mean relative induction of CD54 from three separate experiments is shown. Error bars indicate 1 standard deviation from the mean.

that substitution of a oppositely charged amino acid (R132E) at this point caused reduced induction of CD54 without affecting NF-κB (38). The most marked difference observed in these experiments was greater induction of CD54 by the group D isolates, G50 and G64 (Fig. 6). Group D variants are defined as having 35 amino acid substitutions relative to the B95.8 sequence. Sixteen of these 35 mutations are unique to group D (Fig. 1B). In terms of NF-κB, AP-1, and STAT activation, the group D variants behave the same as the Chinese NPC variants. This suggests that 1 or more of the 16 unique amino acid substitutions may be involved in regulating as yet unidentified signaling events.

DISCUSSION

Much is known about the biological properties of LMP-1 with regard to its oncogenic potential and its effects on cell gene expression, cell survival, cell proliferation, and immunogenicity. There has also been extensive study of the cell signaling pathways transduced by LMP-1 to activate families of transcription factors which mediate the changes in cellular phenotype. Furthermore, constitutive activation of the NF-κB, AP-1, and STAT transcription factors, all of which are activated by LMP-1, is implicated in the development of EBV-associated malignancy (4, 5, 10, 12, 51, 76). However, most of our knowledge of LMP-1 signal transduction and biological functions has been gained from studies on one prototype LMP-1 derived from the B95.8 strain of EBV. Studies with LMP-1 variants have been mainly restricted to analysis of del-LMP-1 NPC-derived variants from two Chinese patients, which have been shown to have greater oncogenic potential in nude mice and may be less immunogenic, and from one North African patient (13, 14, 34, 36, 42, 58, 71). It is not clear to what extent the functions of NPC-derived del-LMP-1 variants are shared by sequence variants from other geographical locations. The present study, in which a panel of nine LMP-1 genes was investigated, is an important development because it represents a more systematic analysis of the abilities of defined groups of European and Chinese variant LMP-1 genes to activate the NF-κB, AP-1, and STAT transcription factors.

The ability of two NPC-derived LMP-1 genes to activate NF-κB more efficiently than B95.8 LMP-1 (Fig. 3) is consistent with previous reports (42, 58). However, that the European group D variants (G50 and G64) also have increased NF-κB signaling is a novel observation. There are just three amino acid substitutions which are common to both group D and the NPC group but not present in any of the other variants, and these are all located in the cytosolic N terminus of LMP-1: R13Q/P, R17L, and L25I (Table 1). The simplest interpretation, which assumes that the mechanism is the same for group D and Chinese NPC variants, is that enhanced NF-κB activity is due to the shared substitutions in the N terminus. The N terminus of LMP-1 is involved in LMP-1 turnover and the association of LMP-1 with the cytoskeleton (2, 73). The N terminus and the transmembrane domain of B95.8 LMP-1, when expressed alone, have inhibitory effects on NF-κB signaling and activity of the LMP-1 promoter (67).

Very recently, Blake et al. reported data that were interpreted as demonstrating that the increased NF-κB signaling of Cao LMP-1 mapped to the transmembrane domains rather than to the N terminus (8). Furthermore, Miller et al. mapped five amino acid changes, in the transmembrane domains of LMP-1, which were implicated in the increased NF-κB activation induced by the C15 North African NPC LMP-1 (58). While the C15 LMP-1 is distinct in many respects from our group B and C variants, four of the five apparently critical substitutions in the C15 variant (I85L, F106Y, I122L, and M129L) are also present in our group B and C variants, which activate NF-κB to levels similar to those seen with B95.8 LMP-1 (Fig. 3B). Furthermore, mutation of each of these four residues in B95.8 LMP-1 failed to ascribe the phenotypic differences to any single residue (8). The remaining change in the C15 LMP-1 variant (E128D) was found in the group D variants but not in the Chinese NPC variants (see Table 1). Interestingly, amino acid 128 is located in a cytoplasmic loop of the transmembrane spanning domain, which has previously been shown to influence the induction of CD54 by LMP-1 (38). However, the significance of the E128D substitution remains to be confirmed experimentally. Together, these data suggest that the efficiency of NF-κB activation can be influenced by more than one type of naturally occurring amino acid substitution in the N terminus and/or the transmembrane domain.

Although AP-1 has not been implicated in such a wide range of biological functions of LMP-1 as has NF-κB, it does appear to be important in an antiapoptotic function of LMP-1 (51) and in the induction of IL-8 by LMP-1 (20). There are reports that LMP-1 variants can activate AP-1 with different efficiencies (8, 14, 25). One study reported that Cao LMP-1 was less efficient than B95.8 LMP-1 at activating AP-1 (14), while a second study from the same laboratory (8) produced the opposite result. Our results with Cao and B95.8 LMP-1 are in agreement with the latter of these two reports (8) and with a study from a third laboratory (25). In addition, we have identified three groups of LMP-1 variants with increased abilities to activate AP-1 (group B, group D, and the NPC variants) relative to those of group A and group C variants (Fig. 4B). Because AP-1 activation by LMP-1 has been mapped only to CTAR2 (19, 45), while NF-κB activation has been mapped to both CTAR1 and CTAR2 (38), one might have predicted that it would be easier to assign substitutions responsible for influencing AP-1 activation. However, this was not the case. Analysis of the sequences of the two sets of LMP-1 variants segregated on the basis of AP-1-activating ability does not obviously implicate any particular substitution(s).

These data highlight the complexity of the signaling mechanisms of LMP-1 and the incompleteness of our current understanding of how LMP-1 signals. The essential core sequences at amino acids 204 to 208 and 379 to 284 involved in binding TRAF, TRADD, and RIP are well defined (27, 39, 45, 66). However, relatively little is known about sequences outside these core domains which, while not essential, can clearly exert a significant influence on the protein interactions necessary for signal transduction. In this context it should be noted that while CTAR1 and CTAR2 are both independently able to activate signals, the effect is not simply additive; there appears to be some physical cooperation between CTAR1 and CTAR2 (26). This cooperation widens the scope for mutations that may indirectly affect the optimum binding conformation of the LMP-1–TRAF–TRADD–RIP complexes. It also raises the possibility that the number of 11-amino-acid repeats in the C ter-

minus could affect any physical cooperation between CTAR1 and CTAR2, although our data do not reveal any simple correlation between the number of repeats and NF-κB or AP-1 activation (see Fig. 2A, 3B, and 4B). The effect of sequence variation on LMP-1 signaling will be the summation of the effects (both positive and negative) on the overall ability to bind and activate signaling protein complexes. If several mutations are contributing to the overall effect, then it will be difficult to pinpoint particular amino acid substitutions as being responsible. However, by defining groups of sequence variations with distinct signaling properties, we can begin to understand how natural sequence variation in LMP-1 may contribute to the development of EBV-induced disease.

In contrast to the highly conserved nature of the core binding domains for TRAF, TRADD, and RIP, several substitutions and deletions in the putative JAK-3-binding domains have been identified by Gires et al. (28) (see Fig. 1). One might predict that these sequence variations would have marked effects on JAK-3 and STAT activation. However, we found the ability of all LMP-1 variants to activate STAT-dependent transcription to be remarkably constant (Fig. 5). This observation appears to be incompatible with the hypothesis that JAK-3 binding to CTAR3 is required for the STAT activation we detect with the GRR(5)-Luc reporter. In addition, we have observed that deletion of the CTAR3 domain does not abolish STAT-regulated reporter transcription (P. Brennan, unpublished data), while point mutations which inactivate the CTAR1 and CTAR2 domains completely abolish STAT-regulated transcription (10). The region of the LMP-1 molecule containing CTAR3 has been shown to be dispensable for EBV-induced transformation (40), yet the constancy of STAT activation among different LMP-1 variants (Fig. 5) suggests an important role for STAT activation. Indeed, STATs are constitutively activated in EBV-associated tumors (12, 76) and are involved in regulating viral gene expression (12). These observations highlight the need for further studies to elucidate the mechanisms of STAT activation by LMP-1.

Analysis of CD54 (ICAM-1) protein upregulation by the LMP-1 variants was included in this study primarily because there is evidence that a novel, as yet unidentified signaling pathway is involved together with NF-κB (56). The most striking result obtained with our panel of variants was that group D variants (G50 and G64) induced 40 to 100% more CD54-positive cells than other LMP-1 variants. This result cannot be attributed simply to differences in NF-κB-, AP-1-, and STAT-regulated transcription, since group D and NPC variants were indistinguishable with regard to these signaling activities but were clearly distinct with regard to their capacity to induce CD54. This provides more evidence in support of a further pathway or pathways that cooperate with NF-κB in the induction of CD54 by LMP-1 (56). Since group D variants have 16 unique amino acid substitutions, there are many possible sequence changes which could be responsible for their uniquely efficient ICAM-1 upregulation.

Interestingly, a recent study showed group D variants to be underrepresented in Hodgkin's disease patients relative to the healthy population (68). It was suggested that a mutation in the ATF-2/CREB site in the LMP-1 promoters of group D variants, which had been shown previously to decrease promoter activity, might decrease LMP-1 expression and thus reduce the likelihood of developing Hodgkin's disease. Although the effect of nucleotide sequence variation in the 5′ regulatory regions of the LMP-1 genes is a potentially important consideration, it is worth noting that we observed no major differences in LMP-1 levels among the spontaneous LCLs from which the LMP-1 variants were cloned (C. A. Fielding, unpublished data). Our new observation with CD54 raises the possibility that the underrepresentation of the group D variants in Hodgkin's disease may reflect a more immunogenic phenotype induced by LMP-1 through induction of higher levels of CD54 and associated cellular genes. In this scenario, group D LMP-1-positive cells may be more effectively eliminated by immune responses, thus limiting the likelihood of malignant cells developing.

The results in this study show that sequence variation in LMP-1 can alter its signaling functions. It is possible, therefore, that sequence variation in LMP-1 could influence the etiology of EBV-associated malignancies. The underrepresentation of certain sequence variants in Hodgkin's disease is consistent with a protective effect that might include enhanced immunogenicity and regulation by immune responses. Other sequence variations could enhance the oncogenic properties of LMP-1, e.g., by enhancing the ability of LMP-1 to promote cell survival through the induction of antiapoptotic proteins. This study has highlighted the fact that the relationship between sequence variation and signaling function is complex to such a degree that it is unlikely that a particular amino acid substitution or deletion will define a disease-associated variant of LMP-1. The way forward in understanding the effects of sequence variation on LMP-1 function lies not in analyzing the effects of single amino acid changes in isolation but in refining the definition of variant groups which correlate with the known functions of LMP-1.

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