JOURNAL OF VIROLOGY, Mar. 1993, p. 1638–1646
0022-538X/93/031638-09$02.00/0

Vol. 67, No. 3

All Three Domains of the Epstein-Barr Virus-Encoded Latent Membrane Protein LMP-1 Are Required for Transformation of Rat-1 Fibroblasts

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LMP-1, the Epstein-Barr virus latent membrane protein 1, is the only protein encoded by the virus that has been shown to have the properties of a transforming oncogene in rodent fibroblasts such as Rat-1 cells. LMP-1 is phosphorylated and proteolytically cleaved in Rat-1 cells in a manner similar to that seen in human lymphocytes. In this study, we demonstrate that all three major domains of LMP-1 (N-terminal, transmembrane, and C-terminal domains) are required for the ability to transform Rat-1 cells in culture, as assayed by loss of contact inhibition. This study is the first demonstration of a functional role for the C-terminal domain of LMP-1. Our analysis suggests that there are at least three distinct regions of the C terminus involved in signalling. Amino acids 306 to 334, which generate a toxic signal in the absence of amino acids 334 to 364, and the last 23 amino acids, 364 to 386, are essential for transformation. Biochemical analysis of the LMP-1 mutants with the three domains deleted indicate that the mutant N-terminal with the domain deleted is phosphorylated normally but is inefficiently cleaved compared with the wild-type LMP-1. The mutant with the transmembrane domain deleted is also phosphorylated but is not cleaved, showing that phosphorylation of LMP-1 does not require membrane association. The nontransforming mutant with the C-terminal domain deleted that lacks the last 23 amino acids is phosphorylated and cleaved. Therefore, these processing events alone are insufficient to generate a transforming signal.

Epstein-Barr virus (EBV) is a human herpesvirus that infects B lymphocytes and certain epithelial cells. EBV is associated with several malignancies including nasopharyngeal carcinoma, most cases of Burkitt's lymphoma, lymphoproliferative disorders in immunosuppressed patients, and over 60% of lymphomas in patients with Hodgkin's disease (for a review, see reference 17). The EBV genome is present in a latent form in these malignant cells. EBV infection of resting human B cells can be studied in vitro. The virus activates these B cells and causes them to become immortalized, latently infected, proliferating lymphoblasts (for a review, see reference 17). This process and the lymphoblastoid cell lines (LCLs) obtained in this manner have been employed as a model system for studying the mechanisms of herpesvirus latency, B-cell activation, and oncogenic transformation. It seems likely that this capacity of EBV to immortalize cells in culture is related to its tumorigenicity in vivo. To date, nine virus-encoded proteins have been detected in latently infected B cells (for a review, see reference 9). These proteins include six nuclear proteins (EBNAs) and three membrane proteins (LMP-1, TP-1, and TP-2). Several of these viral proteins have been shown to be required for the activation and immortalization of infected B cells in vitro and are expressed in immunoblastic types of B-cell lymphoma. The EBNA-1 protein is required for maintenance of the EBV episomal genome and is found in all EBV-infected cells. LMP-1 is the only virus-encoded protein shown to have the function of a transforming oncogene in rodent fibroblast lines, such as Rat-1 (21) and BALB/3T3 cells (2). LMP-1 is found in EBV-associated, non-B-cell malignancies; being detected in more than 60% of all nasopharyngeal carcinoma, and Hodgkin's lymphoma (8). This finding suggests that LMP-1 may play a direct role in the neoplastic transformation of non-B cells in vivo.

The predicted structure and sequence of LMP-1 have been described elsewhere (4, 13). The orientation of LMP-1 in the plasma membrane has been determined in a variety of ways, including immunofluorescence analysis and protease digestions of viable and permeable cells (7, 13, 14). LMP-1 has a short, hydrophilic, cytoplasmic amino terminus (N terminus) approximately 25 amino acids long that lacks a characteristic signal peptide. The N terminus is followed by six hydrophobic, approximately 20-amino-acid α-helical transmembrane segments, separated by five 8- to 10-amino-acid reverse-turn loops. Finally, there is an approximately 200-amino-acid carboxy terminus (C terminus), rich in acidic residues, that projects into the cytoplasm. LMP-1 is known to associate with components of the cell cytoskeleton (15), including the protein vimentin (11). This cytoskeletal association probably occurs via the short cytoplasmic amino terminus of the protein (16, 21). LMP-1 has phosphorylated serine and threonine residues at a ratio of approximately eight phosphoserines to one phosphothreonine (1, 15). This phosphorylation occurs predominantly at serine 313 and threonine 324 in the cytoplasmic C terminus (19). LMP-1 has a very short half-life of 2 to 3 h (1, 15), and we have previously shown that the short half-life is due to specific cleavage of the C terminus of the protein (at leucine 242), which releases a 25,000-molecular-weight fragment (p25) into the cytoplasm and leaves a 35,000-molecular-weight fragment in the membrane (18). p25 is highly negatively charged and has phosphorylated serines and threonines in a ratio similar to that for LMP-1 (18).

Previously published studies have examined deletion mutants of LMP-1 and shown that the N terminus and the

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transmembrane portions of LMP-1 are essential for its transforming ability in rodent fibroblasts (3, 13, 21, 22). However, it was reported (3) that the C-terminal 199 amino acids of the LMP-1 protein could be deleted and LMP-1 retain its transforming capacity, since it was still able to induce anchorage-independent growth in BALB/3T3 cells, as assayed by colony formation in soft agarose.

The results of the functional (3) and biochemical (18) studies were consistent with the interpretation that LMP-1 is synthesized as an inactive preprotein. Deletion of the C terminus, either by cleavage to produce p35 or by mutational deletion, generates the functionally active form. In this study, we demonstrate that the C terminus is required for the transformation of Rat-1 cells by LMP-1 and we have gone on to correlate the functional and biochemical properties of deletion mutants lacking either the N-terminal, transmembrane, or C-terminal domain of LMP-1.

MATERIALS AND METHODS

Cells and antibodies. ER is an EBV-positive LCL derived in our laboratory by transformation with the B95-8 isolate of EBV. Jijoye is an EBV-positive Burkitt's lymphoma obtained from G. Miller. Rat-1 cells were the gift of Robert Weinberg. Lymphocyte lines were maintained in RPMI 1640 containing 10% fetal calf serum (FCS) (JRH Biosciences) and fed three times a week by diluting the cells to 3×10^5 to 5×10^5 cells per ml of medium. Rat-1 fibroblasts were maintained in Dulbecco modified Eagle medium (DMEM)–10% FCS (JRH Biosciences) and propagated by treating the cells with trypsin and diluting the cells 1:30 in new medium twice a week.

The S12 monoclonal antibody is a murine monoclonal antibody of the immunoglobulin G2a isotype raised against a fusion protein between β-galactosidase and the carboxyl half of LMP-1 and has been described in detail previously (14). The S12 antibody and isotype-matched negative control antibody (1A2) were used as protein A affinity-purified antibody in all experiments.

Plasmids. Diagrammatic representations of wild-type LMP-1 and the different deletion mutants used in this study are shown in Fig. 1. Plasmids pNΔ43, pNΔ128, pCΔ155, pCΔ174, and pΔ27-212 were gifts from Bill Sugden and have been described in detail previously (3). All of these plasmids were derived from the pSV2LMP plasmid (2) and thus have the mutant LMP-1 genes under the control of simian virus 40 enhancer and early promoter. The protein encoded by pNΔ43 (known hereafter as NΔ43 [the names of other proteins encoded by plasmids also made by dropping the letter p) initiates at Met-44 of the wild-type LMP-1 protein and thus lacks the cytoplasmic N terminus and first transmembrane domain of the wild-type molecule. The NΔ128 protein lacks the first 128 amino acids of LMP-1 and therefore has a small cytoplasmic N terminus, followed by the last membrane loop of LMP-1 and the entire C-terminal portion of the molecule. The CΔ174 and CΔ155 proteins lack the last 174 and 155 amino acids of LMP-1, respectively. The Δ27-212 protein has the first 26 amino acids of LMP-1, followed immediately by the last 173 C-terminal residues of the wild-type protein, and thus lacks the entire transmembrane portion of LMP-1.

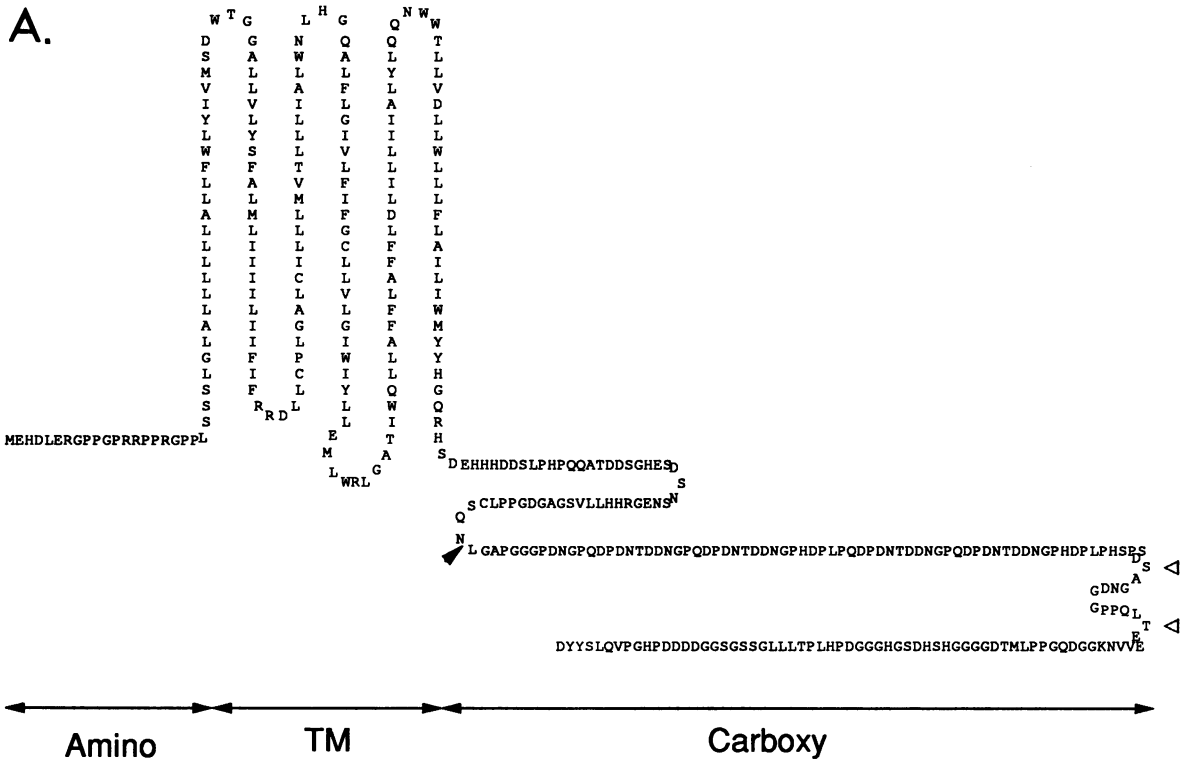
The pSV2gptΔ242-386, pSV2gptΔ306-386, pSV2gptΔ323-386, pSV2gptΔ334-386, and pSV2gptΔ364-386 mutant plasmids were designed by the Amersham oligonucleotide-directed in vitro mutagenesis system with some modifications. The Bluescript phagemid (Stratagene) with the LMP-1 gene inserted in the multiple cloning site at the *Bam*HI site (named pB3LMP-1) was used in place of recombinant M13, as the single-stranded DNA template. The Bluescript phagemid is a plasmid that contains the intergenic region of F1 phage. This region encodes all of the *cis*-acting functions of the phage required for packaging and replication. In *Escherichia coli* with the F-positive phenotype (containing an F′ episome), Bluescript plasmids (including any inserts) are secreted as single-stranded plasmids when the host bacteria are infected by a helper phage. pB3LMP-1 single-stranded DNA was obtained with the R408 phage (Stratagene) and the protocol supplied by Stratagene. Mutant primers were designed to generate new restriction enzyme sites other than *Nci*I to facilitate screening of the final products to identify and isolate mutants. The mutagenesis procedure was performed as detailed in the protocol accompanying the Amersham kit.

Briefly, the mutagenic oligonucleotide was annealed to the single-stranded template and extended by Klenow polymerase in the presence of T4 DNA ligase and a thionucleotide (dCTPαS) to generate a mutant heteroduplex with one phosphorothioate (mutant) strand and one nonphosphorothioate (wild-type) strand. *Nci*I which cannot cleave phosphorothioate DNA was used to generate single-stranded nicks in the wild-type strand of the heteroduplex. Exonuclease III was used to digest away most of the nicked wild-type strand. Exonuclease III digestion times were limited to 15 to 20 min on the basis of the known sequence of the LMP-1 gene and the proximity of the sites to be mutated to the sites nicked by *Nci*I. The mutant strand was then used as a template to reconstruct the double-stranded closed circular molecule, thus creating a homoduplex mutant DNA molecule. Bacterial colonies transformed with this construct were screened by restriction enzyme digestion, utilizing the novel enzyme site engineered into the mutant oligonucleotide. Mutants identified in this manner were subsequently sequenced to confirm the mutated target sequence.

The region of the LMP-1 gene containing the desired site-specific mutation was excised with the *Xho*I enzyme and ligated in place of the identical wild-type LMP-1 fragment in the pSV2gptLMP-1 construct (gift of E. Kieff). The pSV2gptLMP-1 plasmid has been described in detail previously (21). This plasmid has the *gpt* drug resistance marker, read from the simian virus 40 enhancer and early promoter, and the LMP-1-encoding sequence under the control of the metallothionein promoter on the same construct.

Transfections. Transfections of Rat-1 cells were performed by the $CaCl_2$ method (5). Cells were cotransfected with pSV2LMP-derived constructs (pNΔ43, pNΔ128, pCΔ174, pCΔ155, and pΔ27-212) and the pSV2gpt plasmid in a 10:1 ratio or with pSV2gptLMP-1-derived constructs alone (5 μg of plasmid DNA and 5 μg of salmon sperm DNA as carrier per 100-mm-diameter plate). Cells were incubated with the DNA precipitate for 4 to 8 h, washed twice with phosphate-buffered saline (PBS) and then shocked with 2-ml portions of 15% glycerol in PBS for 4 min. The glycerol was removed by two washes of DMEM containing 10% FCS, and the cells were incubated for 24 to 36 h in DMEM–10% FCS prior to drug selection.

Transformation assays. At 24 to 36 h after transfection, the Rat-1 fibroblasts were subjected to drug selection in the following medium: RPMI 1640 containing 10% FCS (JRH Biosciences), the appropriate concentration of mycophenolic acid (Sigma) (working strengths of the different lots varied), 160 μg of xanthine per ml, and 10 μg of hypoxanthine (Sigma) per ml. RPMI 1640 was used instead of DMEM



B.

Designation	Structure	Designation	Structure
Wild Type		Δ242-386	
NΔ43 [1]		Δ306-386	
Δ27-212 [1]		Δ323-386	
CΔ174 [1]		Δ334-386	
CΔ155 [1]		Δ364-386	

FIG. 1. (A) Diagrammatic representation of LMP-1 sequence and proposed structure in the membrane. The sequence and putative secondary structure of the LMP-1 protein are shown. The protein's N-terminal, transmembrane (TM), and C-terminal domains are indicated at the bottom of the panel. The N- and C-terminal domains are thought to project into the cytoplasm. The black arrowhead shows the site on the carboxyl domain where LMP-1 is cleaved, giving the p25 and p35 fragments. The white arrowheads point to the residues identified as the major sites of phosphorylation on the protein (19), namely, the serine at amino acid 313 and the threonine at amino acid 324. (B) Schematic representations of the deletion mutants of LMP-1 used in this study. The mutants supplied by Bill Sugden are indicated by superscript ones.

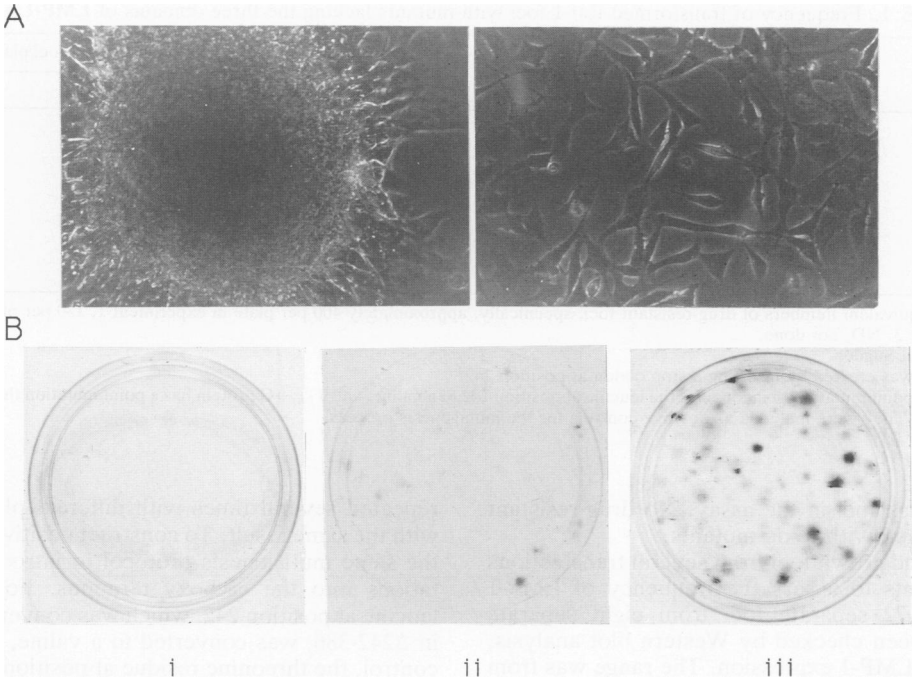


FIG. 2. Appearance of drug-resistant colonies and transformed foci of Rat-1 cells. (A) High-power photographs of a transformed focus of piled-up cells (left) versus an area of nontransformed cells in a colony growing in a monolayer (right). (B) Appearance of drug-resistant colonies and foci on plates after staining with methylene blue. (i) Mock transfected cells (or cells transfected with toxic mutants), (ii) cells transfected with pSV2gpt alone (or a nontransforming mutant), (iii) cells transfected with wild-type LMP-1 (or a transforming mutant) prior to drug selection. Transformed foci stain dark blue, whereas nontransformed colonies stain very light blue.

in order to provide the cells with a slightly richer medium for optimal growth. The medium was changed every 4 days. After 18 to 21 days, drug-resistant colonies grew. The plates were stained with 0.2% methylene blue, and both light-blue normal cell colonies and dark-blue transformed foci of multilayer cells were tallied by the double-blind method. The transformation efficiency (number of foci) was then corrected for transfection efficiency by taking the total number of colonies into account in each case.

Biochemical analyses. For biochemical analyses, Rat-1 fibroblasts were transfected with the appropriate plasmids and subjected to drug selection as described above. After 12 to 15 days, the drug-resistant colonies that arose were picked with cloning cylinders and grown. Expression of the mutant protein was confirmed by Western blot (immunoblot) analysis by the method of Towbin et al. (20) before and after analysis by radiolabeling and immunoprecipitation to confirm that protein expression had not been lost. In all cases, a confluent 100-mm-diameter petri dish of cells (approximately 5×10^6 cells) was gently scraped with a rubber policeman to harvest the cells. Cells were suspended in 50 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (containing 5% β-mercaptoethanol) in a microcentrifuge tube and lysed by ultrasonication for 15 to 30 s with a Kontes 50-W microultrasonic cell disrupter and probe (3.2 by 48 mm). The sample was immediately boiled for 3 to 5 min, any particulate material was pelleted by centrifugation in a microcentrifuge, and the supernatant was used for the Western blot analysis procedure, as described previously (14).

The intracellular localization and phosphorylation status of LMP-1 and p25 were examined by performing immunoprecipitations on fractionated lysates of cells metabolically labeled with ^{32}P, as described previously (15).

RESULTS

All three domains of LMP-1 are required for transformation of Rat-1 fibroblasts. We analyzed the requirement for each of the three major domains of LMP-1 for transformation of Rat-1 cells. We used a transformation assay developed in our laboratory (see Materials and Methods) in which the *gpt* marker was used to select drug-resistant colonies, as described in Materials and Methods. After 18 to 21 days, the plates were stained with methylene blue and transformed foci (areas of multilayer cells that stained dark blue) were counted. Variations in transfection efficiency were corrected for by counting the total number of drug-resistant colonies of monolayer cells on each plate. Figure 2 presents typical results obtained in our transformation assay. Figure 2A shows high-power photographs of a transformed focus of cells growing piled up on other cells versus a nontransformed area of cells growing in a monolayer. Figure 2B shows typical staining patterns obtained with plates containing colonies of Rat-1 cells that were either mock transfected (or transfected with toxic mutants) (panel i), transfected with pSV2gpt alone (or with a nontransforming mutant of LMP-1) (panel ii), or transfected with wild-type LMP-1 (or with a transforming mutant of LMP-1) (panel iii). Transformed foci may be readily detected on these plates as areas of multilayered cells that are stained dark blue compared with nontransformed colonies that stain very light blue and are not readily seen in the photograph. The visible colonies in panel ii of Fig. 2B may represent spontaneous transformants and would

TABLE 1. Frequency of transformed Rat-1 foci with mutants lacking the three domains of LMP-1 protein

Protein	Mutation	No. of transformed foci/plate[a]		
		Expt 1	Expt 2	Expt 3
SV2gpt alone		0.5 ± 0.7	0.33 ± 0.7	0.25 ± 0.5
Wild type	None	31.5 ± 3.5	33.9 ± 8.5	17.25 ± 0.25
NΔ43[b]	N-terminal domain deletion	2.0 ± 0.44	ND	ND
Δ27-212[b]	Transmembrane domain deletion	0.5 ± 0.7	ND	ND
Δ242-386[c]	C-terminal domain deletion	1.5 ± 0.7	0.22 ± 0.44	0.5 ± 0.6
$L_{242}\rightarrow V$	Point mutation[d]	21.0 ± 2.8	30.4 ± 6.1	ND
$T_{340}\rightarrow G$	Point mutation[d]	52.0 ± 4.5	27.4 ± 4.2	16.5 ± 2.9

[a] All constructs gave equivalent numbers of drug-resistant foci, specifically, approximately 400 per plate in experiment 1, 130 per plate in experiment 2, and 70 per plate in experiment 3. ND, not done.
[b] Kindly supplied by Bill Sugden.
[c] The Δ242-386 mutant was created by inserting a stop codon at position 242.
[d] $L_{242}\rightarrow V$ protein has a point mutation that converts the leucine at position 242 to a valine, and $T_{340}\rightarrow G$ protein has a point mutation that converts the threonine at position 340 to glycine. They were included as positive controls for the mutagenesis process.

probably be scored as foci in the assay. No drug-resistant colonies were obtained with toxic mutants.

We have picked and grown foci from several transfections with different mutants to assess the frequency of LMP-1 expression. In all, 72 separate foci from eight separate transfections have been checked by Western blot analysis, with 62 positive for LMP-1 expression. The range was from seven of nine foci positive to all nine foci positive. Given the difficulty we have experienced in maintaining stable expression of LMP-1, we suspect that these numbers represent a minimal estimate with the negative results, in most cases, representing positive foci that lost expression while being grown.

In our initial experiments, we tested the transforming capacity of three mutants each lacking one of the three major domains of LMP-1. For the N-terminal and transmembrane domain-deleted mutants, we employed the NΔ43 and Δ27-212 mutants previously described by Baichwal and Sugden (3). For the C-terminal domain-deleted mutant, we constructed a termination mutant in which amino acids 242 to 386 had been deleted. This mutant was chosen to represent p35—the moiety left in the membrane after cleavage of LMP-1 to release p25. As expected, on the basis of previous studies, neither the NΔ43 nor the Δ27-212 mutant had transforming activity (Table 1). However, the striking result was that the Δ242-386 mutant also lacked transforming ability (Table 2). Since this result was not in agreement with the results of previous studies (3), the experiment was repeated several times with different plasmid preparations with the same result. To construct positive controls, we used the same mutagenesis protocol to introduce irrelevant mutations into the carboxy terminus. For one control, the leucine at position 242, which was converted to a stop codon in Δ242-386, was converted to a valine, and for the second control, the threonine residue at position 340 was converted to glycine. As may be seen in Table 2, both of these mutants transformed as efficiently as wild-type LMP-1.

TABLE 2. Frequency of transformed Rat-1 foci with C-terminal domain mutants of LMP-1

Protein	No. of amino acids deleted	No. of foci/plate[a] (mean ± SD)
SV2gpt alone		0 ± 0
Wild type	0	62.0 ± 2.3
CΔ174	174	0 ± 0
CΔ155	155	0 ± 0
$T_{340}\rightarrow G$[b]	0	42.4 ± 3.4
Δ242-386[c]	145	0.5 ± 0.3

[a] The number of foci in this experiment was normalized to a transfection efficiency of approximately 100 colonies per plate. The results are the averages from two independently transfected plates.
[b] The $T_{340}\rightarrow G$ protein has a point mutation that converts the threonine at position 340 to glycine and was included as a positive control.
[c] The Δ242-386 mutant was created by inserting a stop codon at position 242.

Two trivial explanations for the failure of Δ242-386 to transform were that other mutations were inadvertently introduced into the pSV2gptΔ242-386 plasmid or that the outcome was a function of plasmid construction. The former possibility was unlikely, as the integrity of the mutated sequence had been checked by DNA sequencing. The latter was possible, since Δ242-386 was expressed from the metallothionein promoter with a covalently linked *gpt* resistance marker, whereas previous studies had used the simian virus 40 promoter on the pSV2LMP construct which required cotransfection with pSV2neo (3). To rule out both possibilities, we tested the C-terminal domain deletion plasmids used in the previous study, pSV2CΔ155 and pSV2CΔ174, and were again unable to demonstrate transforming activity in either case (Table 2).

Construction and analysis of a nested set of C-terminal deletions. In the previous section, we demonstrated that an LMP-1 mutant lacking the C-terminal domain was unable to transform Rat-1 cells in our assay. However, we were unable to prove that the mutant protein was actually expressed, since the available monoclonal antibodies to LMP-1 all react with the deleted portion. We therefore constructed a nested series of deletion mutations in order to more precisely map the minimal sequence required for transformation and in the hope of obtaining a deletion mutant that was recognized by monoclonal antibody S12, but was still nontransforming. The results of a typical transformation assay are presented in Table 3. All of the mutants tested did not have transforming activity; however, two, terminating at residues 322 and 333, respectively, were highly toxic. This result was striking and reproducible. For a control for nonspecific effects, we tested independent isolates of the toxic, mutant plasmids prepared in parallel with nontoxic constructs. In no case were drug-resistant colonies ever detected in any transformation assay with these mutants. Of critical importance was the Δ364-386 mutant which also had the nontransforming phenotype; however, this mutant carries the epitope recognized by the S12 monoclonal antibody. Therefore, it was possible to

TABLE 3. Frequency of transformed Rat-1 foci using a nested series of C-terminal domain deletion mutants of LMP-1

Protein	No. of amino acids deleted	No. of foci/plate[a]
SV2gpt alone		1.25 ± 0.84
Wild type	0	24.8 ± 2.7
Δ242-386	145	1.6 ± 1.1
Δ306-386	81	2.2 ± 1.1
Δ323-386[b]	64	0 ± 0
Δ334-386[b]	53	0 ± 0
Δ364-386	23	2.8 ± 1.8

[a] With the exception of Δ323-386 and Δ334-386 (see above), all constructs gave equivalent numbers of drug-resistant foci, approximately 100 per plate.
[b] Δ323-386 and Δ334-386 gave neither foci nor colonies and appear to be highly toxic.

demonstrate directly by Western blotting that the protein was expressed (results not shown; see below). Therefore, we were able to conclude that the last 23 amino acids of the C-terminal domain were required for the transforming function of LMP-1. A summary of all the mutants tested and their phenotypes in the transformation assay is presented in Table 4.

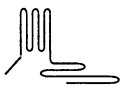
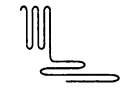
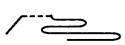
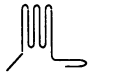
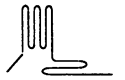
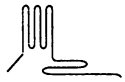
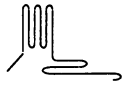
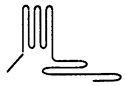
Biochemical analysis of deletion mutants lacking the three essential domains of LMP-1. As discussed above, we have no antibody reagent to detect expression of the Δ242-386 mutant protein that represents the p35 protein left after cleavage of LMP-1 to remove p25. However, the Δ364-386 mutant also has the nontransforming phenotype and carries the epitope recognized by the S12 monoclonal antibody. We therefore were able to biochemically characterize the LMP-1 molecules produced by deletion mutants lacking the three domains essential for transformation, namely, the N-terminal (NΔ43), transmembrane (Δ27-212) and C-terminal (Δ364-386) domains.

The expression of all three mutants was confirmed initially by Western blotting (not shown) and subsequently by immunoprecipitation after ^{32}P radiolabeling, since p25 cannot be detected by Western blotting (18). Figure 3 shows the result obtained with the Δ364-386 mutant. As expected, both the p25 and full-length LMP-1 proteins were truncated because of this deletion and thus migrate with apparent sizes of approximately 22,000 and 55,000, respectively, on a SDS–12.5% polyacrylamide gel. It is apparent that this deletion does not affect phosphorylation or cleavage of the protein. Phosphorylation and cleavage are therefore not sufficient to generate a transformation signal by this deleted form of LMP-1.

The NΔ43 mutant lacks the first 43 amino acids of the wild-type LMP-1 protein, and thus lacks the cytoplasmic N terminus and first transmembrane domain of the protein and cannot associate with the cellular cytoskeleton. The results of our biochemical analysis of the NΔ43 protein are shown in Fig. 4. This mutant is not phosphorylated and can be cleaved, since p25 is detected in the cytoplasmic fraction of the cell expressing the NΔ43 mutant. However, the amount of p25 is severalfold lower than would be expected, considering the amount of the full-size NΔ43 protein, since as we have shown previously (18), ^{32}P-labeled p25 is usually present at a 5- to 10-fold excess over ^{32}P-labeled LMP-1 (see, for example, the ratio in the unfractionated ER LCL control in Fig. 4).

The majority of the NΔ43 protein appears to undergo nonspecific proteolytic degradation, as evidenced by the ladder pattern of specific bands with decreasing size seen in these lanes. We detected identical patterns of bands in more than a dozen independently derived clones of the NΔ43 mutant analyzed by Western blotting. Since the cells were prepared for Western blot analysis by direct lysis in SDS, the degradation of NΔ43 probably does not occur during processing. Moreover, we do not see this pattern in our analysis of wild-type LMP-1 protein. We interpret these results to mean that the N-terminal 43 amino acids of LMP-1 are required for its normal efficient processing in cells by cleavage to release p25. The majority of NΔ43 protein was localized in the membrane, as expected, since NΔ43 possesses five of six transmembrane domains of LMP-1. However, as seen in Fig. 4, about 10 to 20% of the total ^{32}P-labeled NΔ43 signal was reproducibly detected in the cytoplasmic fraction of the cell lysate. Although the reasons for this result are unclear, it was not due to a technical

TABLE 4. Summary of the phenotypes of all of the deletion mutants tested in the transformation assay[a]

Designation	Structure	Phenotype[1]
Wild Type		Transforming
NΔ43[2]		Non-Transforming
Δ27-212[2]		Non-Transforming
CΔ174[2]		Non-Transforming
CΔ155[2]		Non-Transforming
Δ242-386		Non-Transforming
Δ306-386		Non-Transforming
Δ323-386		Toxic
Δ334-386		Toxic
Δ364-386		Non-Transforming

[a] The phenotypes observed in the Phenotype[1] column are shown in Fig. 2B. Mutants provided by Bill Sugden are indicated with superscript twos.

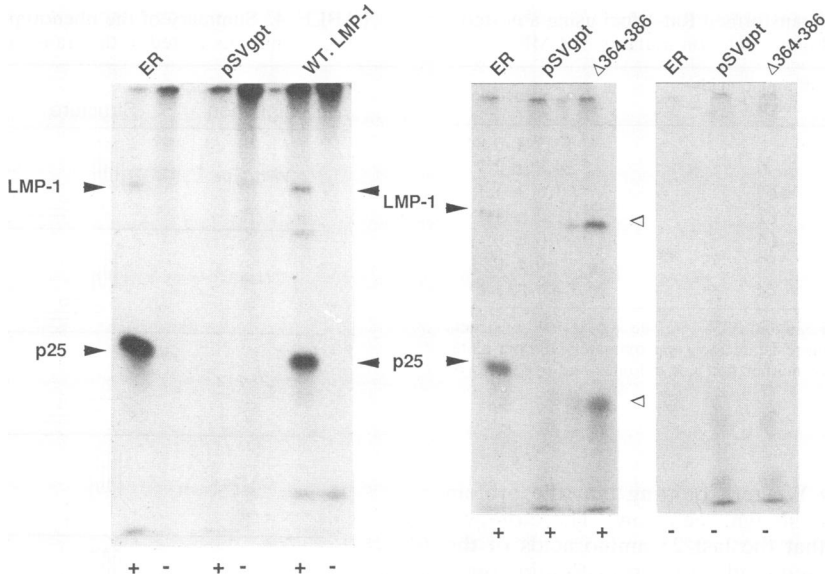


FIG. 3. Biochemical analysis of the Δ364-386 mutant of LMP-1. Rat-1 cells were transfected with the wild-type LMP-1 (WT.LMP-1) or Δ364-386 LMP-1 in the pSV2gptLMP plasmid. For a control, cells were also transfected with pSV2gpt (pSVgpt). Drug-resistant clones were isolated and grown. Clones that were positive for protein expression by Western blotting were labeled with ^{32}P, and immunoprecipitations were performed on the lysates as usual. SDS-PAGE analysis of one of these experiments is shown here. ER is an EBV LCL used as a positive control. pSV2gpt is a negative control, a Rat-1 cell clone transfected with pSV2gpt alone. The labeled black arrowheads denote the locations of LMP-1 and p25, and the unlabeled white arrowheads denote the truncated LMP-1 and p25 proteins, which migrate with apparent molecular weights of 55,000 and 22,000, respectively, on a SDS–12.5% polyacrylamide gel. Lanes: +, precipitated with S12 antibody; −, precipitated with control antibody. Note that the lane between the + lane of pSV2gpt and the + lane of Δ364-386 had no sample loaded, the signal seen is due to spillover from the Δ364-386 lane.

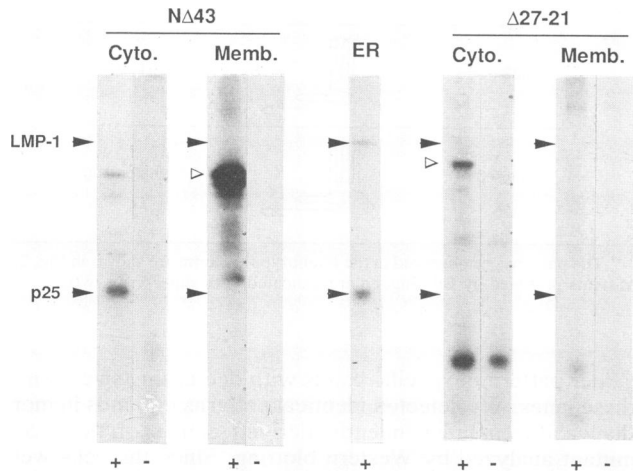


FIG. 4. Biochemical analyses of the NΔ43 and Δ27-212 deletion mutants of LMP-1. Rat-1 cells were cotransfected with pSV2gpt and the pSV2 plasmid construct expressing the NΔ43 or Δ27-212 deletion mutant of LMP-1 at a ratio of 1:10. Drug-resistant clones expressing the mutant proteins were labeled with $^{32}P_i$, and immunoprecipitations were performed on cytoplasm (Cyto.) and membrane (Memb.) fractions with S12 antibody (+) or isotype-matched negative control antibody (−). ER is an EBV-positive LCL used as a positive control. The labeled black arrowheads denote the locations of LMP-1 and p25 on the gels. The unlabeled white arrowheads denote the truncated products of the NΔ43 and Δ27-212 mutant genes. The NΔ43 and Δ27-212 proteins have apparent molecular weights of approximately 55,000 and 50,000, respectively, on a SDS–12.5% polyacrylamide gel.

artifact, since we do not see this effect with wild-type LMP-1 or other membrane proteins.

We were also able to confirm that the NΔ43 mutant does not associate with cytoskeletal elements, since it is readily solubilized in nonionic detergents (not shown). Thus, the NΔ43 mutant of LMP-1 is localized in the membrane, but is not associated with the cytoskeleton; and the NΔ43 mutant is phosphorylated and inefficiently cleaved, releasing p25, which raises the possibility that the inappropriate localization of NΔ43 results in inefficient cleavage.

The Δ27-212 mutant lacks the entire transmembrane sequence of LMP-1 and thus has the first 26 amino acids of LMP-1, followed immediately by the last 173 C-terminal residues of the protein. The results of our biochemical analysis of this mutant are shown in Fig. 4, along with a wild-type control, showing immunoprecipitated ^{32}P-labeled LMP-1 and ^{32}P-labeled p25 from the ER LCL. The Δ27-212 protein migrates with an apparent molecular weight of around 50,000 on the SDS–12.5% polyacrylamide gel. Three observations could be made on the basis of this autoradiograph. First, Δ27-212 is found only in the cytoplasmic fraction. This finding is not surprising, since this protein possesses no transmembrane domains of LMP-1. Second, this protein is phosphorylated, although we cannot be sure that the same sites are used as in wild-type LMP-1. This result suggests that phosphorylation of LMP-1 on its C-terminal domain does not require that this protein be localized to the plasma membrane. Third, no signal for p25 is visible, although most of the cytoplasmic C-terminal domain of LMP-1, including the site of cleavage, is present in this mutant. Even after prolonged exposure of this gel to film, no band corresponding to p25 was detectable. (This band would be expected to migrate at the same position as the ^{32}P-

TABLE 5. Summary of the findings of the biochemical processing and transforming efficiency of mutants with the three major domains of LMP-1 deleted[a]

MUTANT		PI[1]	CLEAVE[2]	TRANSFORM[3]
WT		+	+	+
NΔ43[4]		+	+	-
Δ27-212[4]		+	-	-
Δ242-386				-
Δ364-386		+	+	-

[a] Phosphorylation (Pi^1) was confirmed by immunoprecipitation from cells metabolically labeled with $^{32}P_i$. Cleavage ($CLEAVE^2$) was confirmed by detecting p25 in cells metabolically labeled with $^{32}P_i$. Transformation ($TRANSFORM^3$) was assessed by counting transformed foci in double-blind experiments repeated at least three times. For representative results, see Tables 1 and 2. Mutants supplied by Bill Sugden are indicated by superscript fours. WT, wild type.

labeled p25 in the ER control lane on this gel.) We obtained identical results with the two independently derived Δ27-212 mutants tested. Thus, the Δ27-212 mutant of LMP-1 is phosphorylated, found in the cytoplasm, and does not cleave any detectable p25.

DISCUSSION

Analysis of deletion mutants of LMP-1 allows us to draw a number of conclusions about the properties of this molecule. A summary of the properties of the most relevant mutants is presented in Table 5. All three major domains, the N-terminal cytoplasmic, transmembrane, and C-terminal cytoplasmic domains, are required for LMP-1 to transform Rat-1 cells (NΔ43, Δ27-212, and Δ242-386). Phosphorylation does not require an association with the membrane (Δ27-212) or cytoskeleton (NΔ43). Efficient cleavage to release p25 correlates with and may require association of LMP-1 with the membrane and cytoskeleton (NΔ43 and Δ27-212). Phosphorylation and cleavage are insufficient to generate a transforming signal (Δ364-386). Of these observations, the most surprising was that the C terminus is essential, since this finding was contradictory to those of previous studies (3, 16). All of the C-terminal domain deletion mutants we tested, including one whose expression could be confirmed biochemically, failed to demonstrate a transforming phenotype, so we are sure that this conclusion is correct. The reasons for the discrepancy are unclear but may be due to the use of different assays. Thus, the C terminus may be dispensable for inducing BALB/3T3 cells to form colonies in agarose (3, 16) but essential for the formation of transformed foci by Rat-1 cells. If correct, this implies at least two different types of LMP-1 transformation, one involving the N terminus and transmembrane domain and required in both assays and one involving the C terminus and required only in our Rat-1 assay.

A mutant (Δ242-386) constructed to resemble p35, the moiety that remains in the membrane after cleavage to remove p25, did not have transforming activity. The Δ364-386 mutant, which is cleaved normally and presumably gives rise to a wild-type p35 fragment, also did not have transforming activity. Therefore, p35 is not the active form of LMP-1. Similarly, the NΔ43 mutant becomes cleaved to release p25, yet is also did not have transforming activity, so p25 is not the active form of LMP-1. In our studies, only full-length LMP-1 had transforming activity. This result would seem to rule out the possibility that LMP-1 is synthesized as an inactive preprotein that is activated by cleavage to produce p35 and p25. Instead, our findings suggest that intact LMP-1 is the biologically active form of the molecule and that cleavage serves to inactivate the molecule.

In retrospect, it is not surprising that the C-terminal region of LMP-1 should be essential for transformation. The C-terminal region is approximately 200 amino acids long, representing more than half of the 386-amino-acid LMP-1 molecule, projects into the cytoplasm, has phosphorylated serine and threonine residues, and is specifically cleaved, with a short half-life, to produce the p25 fragment (18).

Our findings on the nested set of C-terminal domain deletions also have implications for the role of the C terminus in the function of LMP-1. Deletion of as few as 23 amino acids from the C terminus of LMP-1 renders it nontransforming. Since this mutant becomes phosphorylated and is cleaved normally, there must be additional events required to generate the transforming signal that require these 23 amino acids, presumably involving an association with other proteins. Within this sequence reside some striking features, including a stretch of four aspartate residues and two tyrosine residues that are the second- and third-last amino acids on the molecule. While there are a large number of aspartate residues along the C-terminal region of LMP-1, the two tyrosines in this sequence are the only tyrosines in the 200-amino-acid cytoplasmic C-terminal domain of LMP-1, the others being in the transmembrane domains and thus probably hidden in the lipid bilayer. As yet there is no evidence for tyrosine phosphorylation on LMP-1, so these two hydrophobic, aromatic residues may contribute to the formation of a unique configuration in the otherwise highly acidic and hydrophilic C-terminal domain.

Also striking was the observation that deletion of 64 or 53 amino acids in mutants Δ323-386 or Δ334-386, respectively, resulted in proteins that were consistently toxic, whereas deletion of more (for example, 81 amino acids [Δ306-386]) or fewer (for example, 23 amino acids [Δ364-386]) amino acids resulted in mutants that simply did not have transforming activity. This result suggests that the sequences around amino acids 306 to 334 somehow are capable of poisoning the cells, but this toxicity is alleviated by the presence of the more-C-proximal sequences. It may not be a coincidence that the major sites of serine and threonine phosphorylation reside in this region, specifically amino acids 313 and 324 (19). The behavior of these mutants again suggests multiple interactions between the C-proximal end of the C-terminal domain and other, presumably cellular, proteins.

LMP-1 normally turns over quickly, or has a short half-life, because of cleavage releasing p25. In our experiments, the Δ364-386 mutant appears to turn over normally, suggesting that the required sequences for rapid turnover are still present. The toxic Δ323-386 and Δ334-386 mutants may lack these sequences but retain major sites of phosphorylation, whereas the nontoxic Δ306-386 mutant would lack both. This is one explanation as to why Δ323-386 and Δ334-386 are toxic, i.e., they may be able to signal but cannot be inactivated by rapid turnover. If correct, this predicts that the sequences required for rapid turnover reside between amino acids 334 and 364.

The N terminus of LMP-1 is required for association with the cellular cytoskeleton, and we have observed that the NΔ43 mutant is inefficiently cleaved to produce p25. Thus, in addition to an absolute requirement for membrane association, as demonstrated with the Δ27-212 mutant, cytoskeleton association also appears necessary for efficient cleavage and turnover. This finding is consistent with the previous reports that both the NΔ43 and Δ27-212 mutants turn over slowly along with bulk cellular proteins (16) and further supports the idea that rapid turnover is due to specific cleavage. In the absence of this mechanism, the protein turns over more slowly because of nonspecific degradation. Since NΔ43 and Δ27-212 are both nontransforming and nontoxic (6), the cell would not be adversely affected by their slow turnover.

Although LMP-1 has no obvious sequence homology to known proteins, it has several structural features in common with the β-adrenergic receptor and rhodopsin family of receptors. These similarities include regulation through serine and threonine phosphorylation, an extensive cytoplasmic C-terminal domain, and multiple transmembrane sequences (10). The major difference is that LMP-1 lacks the initial transmembrane domain seen in these proteins, and consequently, LMP-1 has an intracellular rather than extracellular N terminus. LMP-1 associates with the cytoskeleton via its intracellular N terminus, and this association is essential for transmitting the transforming signal to the cell. An attractive hypothesis is that if LMP-1 were to have an extracellular N terminus, LMP-1 could be a receptor for a growth factor and transmit a growth signal to the cell upon binding of the factor. Loss of the extracellular N terminus and subsequent direct association of LMP-1 with the cytoskeleton via its N terminus may result in the protein constitutively transmitting its growth transformation signal to the cell, without the need for ligand binding. Thus, LMP-1 may behave as a truncated, constitutively active form of a growth factor receptor. The observation that LMP-1 is rapidly inactivated through cleavage, with a half-life of 2 to 3 h further suggests that it may deliver its signal in a cell cycle-regulated fashion.

In conclusion, studies on deletion mutants of LMP-1 point to a complex series of interactions involved in generating the growth signal. The N-terminal sequences are required for cytoskeletal association, and the transmembrane sequences are required for membrane association. Without these sequences, the protein is inert. Therefore, these sequences must allow the correct positioning of LMP-1 in the membrane so that the C terminus can be active. Our results suggest that at least three distinct regions of the C terminus are involved in generating the transforming signal. Amino acids 306 to 334 contain the major sites of phosphorylation that generate a toxic signal in the absence of amino acids 334 to 364 which are required for rapid turnover. Amino acids 364 to 386 are also essential.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AI-18757 and CA-31893.

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