

Latent Membrane Protein 1 Is Critical for Efficient Growth Transformation of Human B Cells by Epstein-Barr Virus¹

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ABSTRACT

The EBV latent membrane protein 1 (LMP1) is an integral membrane protein that acts like a constitutively activated receptor. LMP1 interacts with members of the tumor necrosis factor receptor-associated factor family, as well as with tumor necrosis factor receptor-associated death domain, resulting in induction of nuclear factor- κ B, the p38 mitogen-activated protein kinase pathway, and the c-Jun NH₂-terminal kinase activator protein 1-signaling cascade. The binding of Janus kinase 3 results in activation of signal transducers and activators of transcription. The domain structure of LMP1 has been mapped extensively, but the quantitative contribution of distinct LMP1 domains to the efficiency of B-cell proliferation by EBV has not been determined. On the basis of the maxi-EBV system, which allows us to introduce and study mutations in the context of the complete EBV genome, a panel of 10 EBV mutants with alterations in the LMP1 gene locus was established. The mutant EBVs were tested for their efficiency to induce and maintain proliferation of clonal B-cell lines *in vitro*. Surprisingly and with reduced frequency, EBV mutants which deleted LMP1's COOH terminus, transmembrane domains, or the entire open reading frame were able to generate proliferating B-cell clones that were dependent on the presence of human fibroblast feeder cells. A B-cell clone carrying the LMP1-null mutant EBV genome was also analyzed for oncogenicity in severe combined immunodeficiency mice. Our results demonstrate that LMP1 is critical but not mandatory for the generation of proliferating B cells *in vitro*. LMP1 functions greatly contribute to EBV's transformation potential and appear essential for its oncogenicity in severe combined immunodeficiency mice.

INTRODUCTION

EBV is a human herpes virus identified as the first human tumor virus (1). It is associated with human malignancies, including Burkitt's lymphoma, Hodgkin's disease, and nasopharyngeal carcinoma, among others. *In vitro* EBV predominantly infects resting primary human B-lymphocytes, which can acquire the characteristic of unlimited cell proliferation, giving rise to LCLs.³ This process partly reflects the pathogenetic mechanisms of tumor development. In growth-transformed B cells, only 11 of the 80 viral genes are expressed. Among them, 5 genes, the nuclear antigens EBNA1, EBNA2, EBNA 3a and c, and the LMP1, are considered to be essential for cellular transformation (see Ref. 2 for a recent review). LMP1 is the only protein that is transforming in nonlym-

phoid cells, such as rodent fibroblasts (3–5), and transgenic mice indicate that LMP1 is likely to be a main factor in EBV-associated lymphomas (6, 7). In B cells, LMP1 mimics the phenotypic effects of an activated CD40 receptor, which include sustained cell proliferation, expression of adhesion molecules, and secretion of antibodies (8–10). In CD40 knockout mice, however, LMP1 only partially substitutes for CD40's functions (10).

LMP1 is an integral membrane protein composed of a short cytoplasmic NH₂ terminus of 24 aa, six transmembrane spanning domains of 162 aa, and a cytoplasmic COOH terminus of 200 aa. It has been shown that LMP1 acts as a constitutively active pseudo receptor (11). For LMP1's ligand-independent activity, the six transmembrane domains are critical in mediating the spontaneous aggregation of the molecule in the plasma membrane (11–13). The cytoplasmic NH₂ terminus of LMP1 seems to be essential for the correct insertion of LMP1 in the plasma membrane, interacts with the cytoskeleton (14–16), and helps to localize LMP1 in microdomains or rafts (Refs. 12, 17, and references therein), which are known to be hot spots for signal-transducing molecules. LMP1's cytoplasmic COOH terminus interacts with TRADD (18, 19), as well as with members of the TRAF family (20–25), indicating its role in signaling. Apart from NF- κ B (26, 27), LMP1 induces the p38 MAPK (28, 29) and JNK1/AP1 (19, 30) pathways. Two subdomains of LMP1, called CTARs 1 and 2, are responsible for signal induction for both NF- κ B and MAPK pathways and have been proposed to be critical for cellular transformation (4, 18, 26, 31). A third domain, CTAR3, located between CTAR 1 and CTAR 2, triggers the JAK3/STAT signaling cascade (32). The role for CTAR3 is controversial (33, 34).

In the context of EBV, LMP1 is thought to be essential to induce and maintain cellular transformation of B cells *in vitro* based on genetic analyses, but it is still not obvious which domains of LMP1 are really critical, because most of the examined LMP1 mutants did not take LMP1's fine structure into account (14, 31, 34–37). On the basis of the maxi-EBV system (38), it is now feasible to assess the contributions of the different domains to the process of B-cell proliferation quantitatively. Toward this aim, a panel of EBV mutants with alterations within the *LMP1* gene was established, and their effect on the *in vitro* frequency of B-cell growth transformation was measured clonally. To our surprise, even a mutant EBV lacking the entire *LMP1* gene was capable of fostering proliferation of B-cell clones, although at dramatically reduced frequency and only in the presence of irradiated fibroblast feeder cells. This particular mutant was also analyzed for its oncogenicity in the SCID mouse model (Ref. 39 for a review). Our data indicate that LMP1's functions contribute markedly to B-cell proliferation and are essential for EBV's oncogenic potential in SCID mice.

MATERIALS AND METHODS

Cells. The 293 cell line is a human embryonic epithelial kidney cell line; Raji is an EBV-positive human Burkitt's cell line. B95.8 is an EBV-positive lymphoblastoid marmoset B-cell line. 721 is a human B-cell line immortalized with the B95.8 strain of EBV. WI38 are primary human fibroblasts obtained from American Type Culture Collection. The cell lines are described as well

Received 7/9/02; accepted 3/31/03.

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¹ This work was supported by the Sonderforschungsbereich SFB455, additional grants from the Deutsche Forschungsgemeinschaft, Public Health Service Grants CA70723 and CA22443, and by institutional grants.

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³ The abbreviations used are: LCL, lymphoblastoid cell line; LMP1, latent membrane protein 1; NF- κ B, nuclear factor- κ B; TRADD, tumor necrosis factor receptor-associated death domain; JNK, c-Jun NH₂-terminal kinase; MAPK, mitogen-activated protein kinase; JAK, Janus kinase; GFP, green fluorescent protein; CTAR, COOH-terminal activator region; AP, activator protein; SCID, severe combined immunodeficiency; ATF, activating transcription factor; TRAF, tumor necrosis factor receptor-associated factor; STAT, signal transducers and activators of transcription; aa, amino acid; SH3, Src homology 3.

Table 1 *LMP1 mutants*

Overview of the LMP1 mutants in different maxi-EBVs, details of the individual mutations, and their expected phenotype deduced from published data and number of “green Raji units” needed to establish a single proliferating B cell clone.

LMP1 mutants in maxi-EBVs	Domain	Mutation	Expected phenotype	“Green Raji units” per B cell clone
ΔPxQxT-LMP1	CTAR1, TRAF binding	ΔPxQxT (aa 204–208)	NF-κB, MAPK/ATF, p38 signaling affected	47.3
Y384G-LMP1	CTAR2, TRADD binding	aa exchange Y384G	NF-κB, p38, MAPK/ATF, JNK/AP1 signaling affected	58.2
ΔJAK3-LMP1	CTAR3 JAK3 binding	Δ 33 bp repeat (aa 250–307)	JAK3 and STAT activation impaired	5.2
LMP1:CD40	COOH terminus replaced	LMP1 aa 1–191 & CD40 aa223–280	Constitutive CD40 activity	8.7
ΔC-LMP1	COOH terminus deleted	Δ aa 191–386/His-tag	Failure to signal via NF-κB, p38, MAPK/ATF, JNK/AP, and JAK3/STAT	188
P9-10-15A-LMP1	NH ₂ terminus aa 9–15	P9A/P10A/P15A	Localization to cytoskeleton and rafts affected, SH3 element destroyed	9.4
P12-15-16-19-20A-LMP1	NH ₂ terminus aa 12–20	P12A/P15A/P16A/P19A/P20A	Ditto	4.5
ΔTM-LMP1	Transmembrane domain	Δ aa 26–212	No interaction with the cytoskeleton or oligomerization, no constitutive activity	975
Δlytic LMP1	Intron 1 deleted	Δ lytic LMP1 promoter	Loss of dominant negative “lytic” LMP1	5.2
ΔLMP1	LMP1 deleted	Δ aa 1–384	No B cell immortalization	442
wt EBV/2089		None	Wild-type B95.8	4.6

as the purification of primary B lymphocytes from adenoids or umbilical cord blood (40, 41).

Recombinant Maxi-EBV Plasmids. LMP1 mutations (Table 1) were introduced into the plasmid p2167.1 (Fig. 1A). This plasmid also carries the selection marker for kanamycin phosphotransferase in between the *BALF1* gene and the promoter of LMP2A. Derivatives of p2167.1 with the different LMP1 mutant alleles served as shuttle constructs to introduce the mutations into the context of the maxi-EBV plasmid p2089. In p2167.1, all LMP1 mutants are under the control of the LMP1 wild-type promoter. Detailed cloning strategies and information about intermediate plasmids are available on request. All LMP1 mutant plasmids were carefully checked for composition, and relevant sections were sequenced.

Generation of Mutant Maxi-EBV Plasmids. The linearized fragments obtained from the p2167.1 derivatives were transformed into *Escherichia coli* DH10B, as illustrated in Fig. 1B. For double selection of recombinant plasmids, 30 μg/ml chloramphenicol and 30 μg/ml kanamycin were used. Mutant maxi-EBV plasmids were used to establish virus-producing 293 cell lines as described (42).

Production of Infectious Particles. 293 cell clones carrying different maxi-EBV plasmids were transfected with expression plasmids for BZLF1 (43), BALF4 (42), and histone acetyl transferase p300 to induce the lytic cycle. At 72-h postinfection, supernatants were harvested and filtered through 1.2-μm pore size filters.

Quantitation of Virus Titers and Infection of Primary B Cells. The different virus stocks were quantitated via GFP expression of Raji cells infected with defined amounts of the virus stocks to be analyzed. Raji cells (3×10^5) were incubated at 37°C in 24-well cluster plates and cultivated for 3 days to allow expression of GFP. Infected Raji cells remain latently infected and do not express lytic genes, such as gp350/220 (42), indicating that no progeny virus is released under these conditions.⁴ After 3 days, the percentage of GFP-positive cells was determined by UV microscopy. On the basis of these data, “green Raji units” per ml were calculated as a measure of the concentration of infectious maxi-EBV particles in different virus stocks. Primary human B lymphocytes, purified from adenoids or umbilical cord blood (41), were infected with different dilutions of the adjusted virus stocks. In combination with γ-irradiated WI38 fibroblasts as feeder layer, 1×10^5 B cells were plated in a volume of 100 μl/well in a 96-well cluster plate and fed once a week. After 4–6 weeks, the percentage of proliferating cell clones was determined, and several clones were expanded in suspension. As an alternative method, B cells were cultivated in soft agar after infection with the adjusted virus stocks as described (44).

Western Blot Analysis and Immunostaining. Proteins were extracted and analyzed as described (22). Antibodies directed against LMP1’s COOH terminus (CS1–4; DAKO), LMP1’s NH₂ terminus (OT22CN), and EBNA2 were used for detection. Immunoblots were developed using peroxidase-coupled secondary antibodies (Promega) and ECL reagent (Amersham-Pharmacia).

Immunostainings with a monoclonal antibody directed against gp350 was done as described previously (40).

Mice. Eight-week-old SCID mice (Fox Chase) were injected i.p. with 3×10^6 , 3×10^7 , or 1×10^8 cells for each LCL. Mice were sacrificed when clinical signs of illness were observed.

RESULTS

Generation of Recombinant EBVs. The complete genome of the B95.8 strain of EBV was cloned onto a mini F plasmid that also carries expression cassettes for GFP and hygromycin phosphotransferase as phenotypic markers for eukaryotic cells and chloramphenicol acetyl transferase for selection in *E. coli* (38). This plasmid called maxi-EBV plasmid or p2089 (virion particles derived from p2089 are termed 2089) allows the introduction of any mutation in *E. coli* by a procedure outlined in detail in Fig. 1.

We generated 10 LMP1 mutant alleles in the context of the maxi-EBV plasmid p2089. Conventionally engineered derivatives of the shuttle plasmid p2167.1 (Fig. 1A) were constructed, each carrying a specific LMP1 mutant (Table 1) under the control of LMP1’s own promoter. Linear fragments of the p2167.1 derivatives were introduced into the wild-type p2089 maxi-EBV plasmid via homologous recombination in *E. coli* (Fig. 1). After recombination, the resulting maxi-EBV plasmid DNA was isolated and checked by restriction analyses, Southern blot hybridizations, and partial DNA sequencing. Virus production of maxi-EBVs is based on 293 cells that stably carry the mutant maxi-EBV genome. To this end, the maxi-EBV plasmid DNAs were prepared from *E. coli* and transfected into 293 cells, and hygromycin-resistant and GFP-positive single cell clones were selected and expanded as described (38, 40, 42, 45). To confirm the identity and correct genotype of the maxi-EBV-carrying cell lines, total cellular DNA was analyzed by Southern blot hybridizations with appropriate probes (data not shown). For each maxi-EBV mutant, several single cell clones were tested for their ability to support the lytic cycle of EBV. The cell clones were transiently transfected with expression plasmids encoding the genes and transactivators BZLF1, BALF4, and p300. Although BZLF1 acts as the molecular switch to promote the onset of EBV’s lytic cycle (Ref. 2 for a review), p300 improves the inducibility of the lytic mode,⁵ and concomitant BALF4 expression markedly augments virus production (45). Three days after transfection, the supernatants were used for infection of the human B-cell line Raji to determine the number of infectious viral particles. Because these cells become only latently infected and have not been

⁴ U. Dirmeier, B. Neuhierl, E. Kilger, G. Reisbach, M. L. Sandberg, and W. Hammerschmidt, unpublished data.

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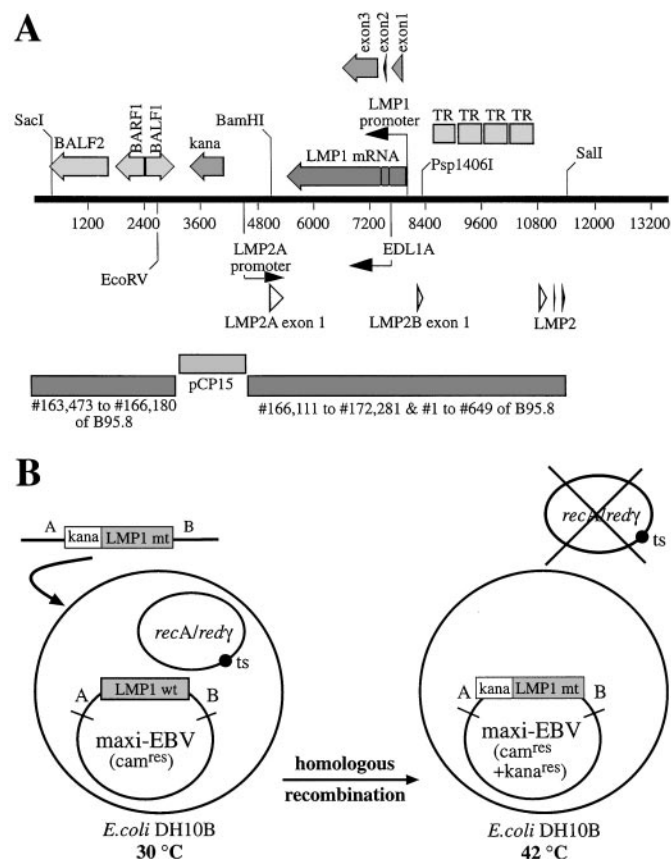


Fig. 1. Construction of mutant LMP1 alleles. In A, plasmid map of the shuttle plasmid p2167.1 encompasses B95.8 sequences as indicated. Immediately downstream of BALF1, a pCP15 fragment carrying the gene for kanamycin resistance was introduced. The complete LMP2A promoter was reconstructed by insertion of a fragment downstream of the pCP15 insert. Additionally shown are the genes for BARF1 and BALF2, exons of LMP2A or B, the terminal repeats (TR), and the promoters for LMP1, EDL1A (lytic LMP1 promoter), and LMP2A. In B, the diagram shows the *E. coli* strain DH10B carrying a chloramphenicol-resistant maxi-EBV plasmid and the ampicillin-resistant plasmid p2650 with a temperature-sensitive replication origin (*ts*) encoding RecA and the exonuclease V inhibitor Red γ to facilitate homologous recombination and transformation with linear DNA fragments. The DNA fragment representing a linearized derivative of p2167.1 contains the LMP1 mutation flanked by two homologous regions A and B and the marker kanamycin resistance (*kana*). After transformation of this fragment into competent *E. coli* cells and homologous recombination, colonies were selected in the presence of chloramphenicol and kanamycin at 42°C.

observed to release progeny virus on superinfection with maxi-EBVs (data not shown), GFP-positive Raji cells allow direct assessment of the concentration of infectious EBVs. This information was used to adjust the virus stocks such that identical numbers of infectious maxi-EBV particles could be used for subsequent infection of primary human B cells.

LMP1 Mutant Alleles Promote Proliferation of Primary Human B Cells. To identify domains of LMP1 critical for EBV's growth transforming potential, 10 EBV mutants in LMP1 were established (Table 1). The mutations or deletions in the COOH terminus of LMP1 were expected to affect the induction of the NF- κ B, MAPK/ATF, p38, JNK/AP1, and JAK3/STAT signaling pathways, and the complete deletion of the COOH terminus should result in LMP1's failure to signal. Two NH $_2$ -terminal LMP1 mutants were included to determine the contribution of a number of proline residues within a potential SH3 motif in this domain of LMP1. Because LMP1 and CD40 are known to share analogous functions, we were curious to learn if a constitutive CD40 signal could replace LMP1's contribution to cellular proliferation; this possibility was assessed by the LMP1:CD40 chimeric molecule (11). During the early phase of EBV infection, an additional, truncated form of LMP1 (aa 129–386) is ex-

pressed, termed "lytic" LMP1, that might compete with signaling of full-length LMP1 as proposed (46). To evaluate the role of lytic LMP1, the promoter of the lytic LMP1 was deleted. A drastic alteration of LMP1 is the deletion of its entire transmembrane domain, resulting in a cytoplasmic location of the truncated LMP1 gene product (47). This Δ TM-LMP1 mutant should be nonfunctional because LMP1's localization and oligomerization in the membrane is expected to be essential for the activity of the molecule (11, 12). Finally, a complete deletion of the entire coding region of LMP1 was introduced, resulting in the maxi-EBV Δ LMP1.

Infection of primary B lymphocytes with EBV results in so-called growth-transformed B-cell lines. This process has been shown to follow one-hit kinetics (44), indicating that one biologically active EBV particle is sufficient to yield a single growth-transformed B-cell clone 4–6 weeks after infection of resting primary B lymphocytes (44, 48). Infection of primary B cells does not yield infectious progeny virus but results in a robust and latent viral infection in the growth-transformed cells (Ref. 2 and references therein), whereas uninfected primary B lymphocytes die within days in cell culture. Therefore, the efficiency of growth transformation of primary B cells is not perturbed by progeny virus multiplying and spreading in the assay, giving rise to multiple rounds of infection.

Dilutions of the adjusted virus stocks of wild-type 2089 and mutant maxi-EBVs were used for infection of primary human B cells in 96-well plates on irradiated human fibroblast feeder cell layers as described (48, 49). Fibroblast feeder cells were used to improve the clonal expansion of EBV-infected B cells as described (Ref. 44 and references therein). Potential virus production in primary B lymphocytes infected with the different virus stocks was analyzed by immunostaining for gp350/220 several days after infection. Expression of the glycoprotein gp350/220, a structural component of the EBV particle, would be indicative of the completion of the lytic productive phase of EBV's life cycle (2, 42). No gp350/220 protein could be detected as expected (data not shown).

Infection of primary B lymphocytes with wild-type EBV results in the outgrowth of proliferating B cells. After 6 weeks, the ratio between the number of wells containing proliferating B-cell clones and wells without proliferating cells was determined with virus dilutions, which met the statistical requirements of one-hit kinetics (50). In addition, similar experiments were performed in which infected B cells were cultivated in soft agarose with feeder cell layers (44, 48). The results were calculated as the number of virus particles needed to establish a single proliferating B-cell clone 6 weeks after B-cell infection expressed as "green Raji units" per cell clone (Table 1). Alternatively, the number of green Raji units required for the wild-type maxi-EBV 2089 was set to 100%, and the ratio of a particular mutant to yield a proliferating B-cell clone in relation to wild-type 2089 EBV was expressed as "efficiency percentage of growth transformation in the presence of feeder cells" (Fig. 2). To exclude any background problems, only experiments were included in this study in which mock-infected B-cell preparations were free of contaminating EBV field strains after 6 weeks of cultivation.

Unexpectedly, all LMP1 mutant EBVs gave rise to proliferating B-cell clones 6 weeks after infection, although with very different frequencies (Fig. 2). The first group of mutants showed an efficiency comparable with maxi-EBV 2089 encoding wild-type LMP1. To this group belonged one of the NH $_2$ -terminal mutants (P12-15-16-19-20A-LMP1), the deletion of the lytic LMP1 gene (Δ lytic LMP1), and the 33-bp repeat deleted LMP1 (Δ JAK3-LMP1). The second NH $_2$ -terminal mutant (P9-10-15A-LMP1) and LMP1:CD40 chimera were slightly compromised in comparison with 2089 wild-type stocks and showed ~50% reduction. The two COOH-terminal mutants Δ PxQxT-LMP1 and Y384G-LMP1 were more affected and showed a reduced efficiency of

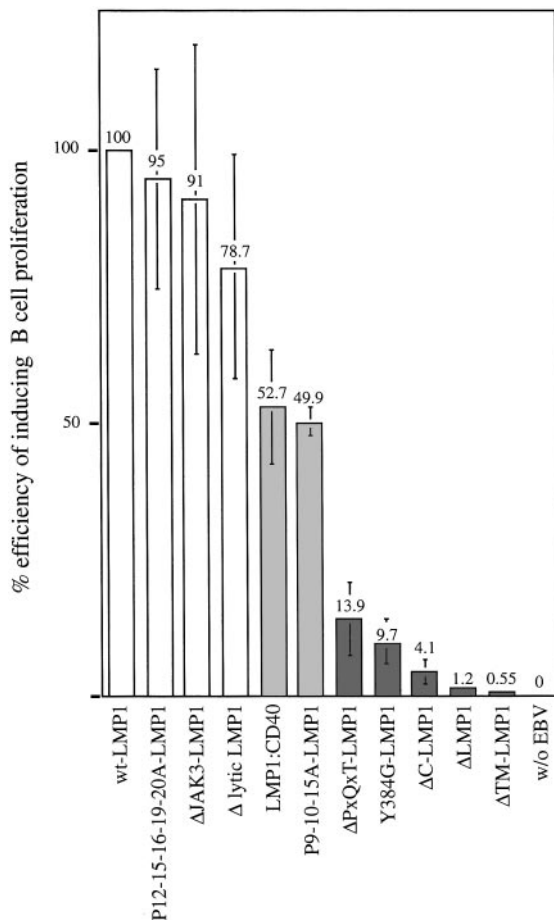


Fig. 2. Transformation efficiency of LMP1 mutant alleles. The efficiencies of inducing long-term B-cell proliferation in the presence of human WI38 fibroblast feeder cells of the 10 different maxi-EBVs based on the number of "green Raji units" (Table 1) were normalized to the wild-type maxi-EBV 2089, which was set to 100%. The data correspond to the mean values of at least three independent experiments and provide SD as indicated. For experiments with the mutant ΔLMP1 and ΔTM-LMP1 maxi-EBVs, the SD was ± 0.37 and ± 0.16 , respectively. Mock-infected B cells (w/o EBV) did not yield any growth-transformed cells.

13.9 and 9.7%, respectively. The mutant virus ΔC-LMP1, the mutant lacking the entire transmembrane region of the *LMP1* gene (ΔTM-LMP1), and the complete LMP1 knockout (ΔLMP1) were severely affected. They showed an impaired efficiency of cellular transformation between 4.1 and 0.55% in contrast to 2089 maxi-EBV, *e.g.*, ≤ 100 -fold more maxi-EBV particles were needed to yield a single proliferating B-cell clone with the maxi-EBV ΔLMP1. The ΔTM-LMP1 maxi-EBV mutant was affected the most because its growth transformation potential was consistently lower than maxi-EBV ΔLMP1.

LMP1 Mutant B-cell Lines. About 20 proliferating B-cell clones of each of the 10 mutant EBVs obtained from infections of primary B cells were expanded and cultivated further for detailed analysis. According to the conditions of end point dilution, the majority of these proliferating B-cell cultures was monoclonal. All proliferating clones could be expanded in the absence of irradiated fibroblast feeder cells with the exception of B cells infected with EBV mutants ΔC-LMP1, ΔLMP1, and ΔTM-LMP1, which could only be expanded successfully in the presence of fibroblast feeder cells. Under those conditions, even these B-cell lines could be cultivated for ≤ 8 months postinfection equal to ~ 40 – 60 population doublings. They could be frozen viably, thawed, and cultivated further without any noticeable problems.

To characterize all expanded B-cell lines, the clonal identity of the proliferating B cells and integrity of the maxi-EBV DNA were care-

fully assessed. Cellular DNAs of all these B-cell clones of each mutant EBV were analyzed by Southern blot analysis, and the mutated LMP1 loci were PCR amplified and investigated via restriction analysis and DNA sequencing (data not shown). The expression of mutant LMP1 proteins in the expanded B-cell clones was analyzed by Western blot detection with various antibodies (examples are depicted in Fig. 3). All mutant LMP1 proteins that could be easily detected by antibodies directed against LMP1's COOH terminus showed the expected migration pattern, and their expression levels are in the range of wild-type LMP1 (Fig. 3A). The ΔC-LMP1 protein could be detected via its His-tag (data not shown) or a LMP1 antibody directed against LMP1's NH₂ terminus; its expression appeared to be heavily up-regulated (Fig. 3B). The LMP1 mutant protein in proliferating B-cell clones obtained with ΔJAK3-LMP1 or the LMP1:CD40 maxi-EBVs could not be detected by the NH₂ terminus-specific antibody, nor was it possible to visualize the proteins with antibodies directed against the COOH terminus of LMP1 or CD40. In those cases, Southern blot analysis of cellular DNAs and sequencing of the LMP1

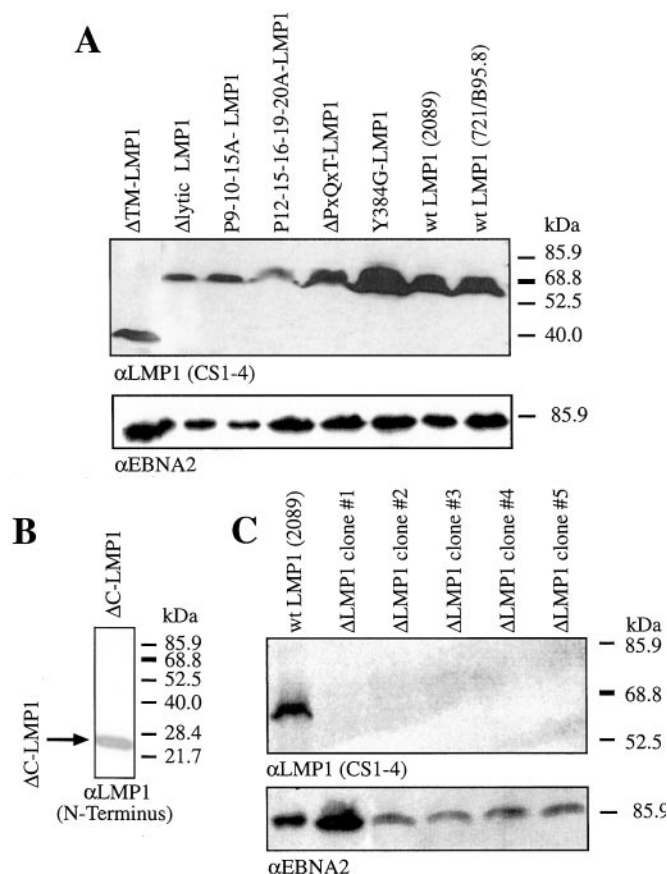


Fig. 3. Detection of mutant LMP1 proteins in EBV-infected and proliferating B cells. By Western blot immunostaining, expression levels and migration patterns of different LMP1 mutants in individual proliferating B-cell clones were analyzed. In A, the expression of mutant LMP1 proteins was detected by a mixture of monoclonal antibodies directed against LMP1's COOH terminus (αLMP1 CS1-4) and as an internal control with an EBNA2-specific antibody. The LCL-labeled "wtLMP1 (2089)" was obtained with the maxi-EBV 2089, whereas the immortalized B-cell line 721 resulted from infection with B95.8 virus. Both cell lines express wild-type LMP1 at the same level, indicating that 2089 maxi-EBV adequately reflects the situation of B95.8-immortalized B cells. In B, because ΔC-LMP1 cannot be recognized by the COOH-terminal LMP1 antibody, this mutant was detected via the antibody OT22CN directed against LMP1's NH₂ terminus. Because the ΔC-LMP1 protein in proliferating B cells infected with the ΔC-LMP1 maxi-EBV seems to be expressed at a very high level, it could be easily detected by the NH₂-terminal antibody. In C, five B-cell clones obtained after infection with the ΔLMP1 maxi EBV were analyzed for expression of EBNA2 and LMP1 by Western blot. As expected, no LMP1 could be detected in these five cell lines, but EBNA2 was readily present.

loci in the B-cell cultures were compatible with the correct expression of the LMP1 mutant proteins. As expected, we failed to detect any LMP1 protein in B-cell clones infected with maxi-EBV Δ LMP1 lacking the complete *LMP1* gene. All B-cell lines obtained with this particular maxi-EBV were EBV positive by Southern blot analysis (data not shown) and expressed EBNA2, excluding a putative contamination with LCLs expressing wild-type LMP1 (Fig. 3C). These results suggested that even a mutant EBV lacking LMP1 entirely is capable to induce B-cell proliferation in the presence of fibroblast feeder cells, although with reduced efficiency.

The phenotypes of many different B-cell clones containing wild-type or mutant LMP1 maxi EBVs were analyzed further by FACS analyses for different surface markers, such as IgG, IgM, intercellular adhesion molecule, CD11a, CD69, CD80, CD83, and others. Unexpectedly, single cell clones differed remarkably in their expression levels when infected with wild-type maxi-EBV 2089 (data not shown), even when obtained from one donor. A similar range of heterogeneity was observed with cell clones infected with LMP1 mutant EBVs, which was therefore not informative. The growth-transformed B cells infected with wild-type or mutant maxi-EBVs adhered tightly to fibroblast feeder cells and, in general, showed an appreciable self-aggregation when cultivated on plastic, only. B cells infected with LMP1 mutant EBVs, which were hardly affected in terms of their growth-transforming potentials as shown in Fig. 2, had a tendency to form bigger clumps similar to B cells infected with wild-type EBV 2089. The doubling time was much prolonged ≤ 4 days with the EBV mutants Δ C-LMP1, Δ TM-LMP1, and Δ LMP1, in contrast to cells infected with wild-type EBV, which showed an estimated doubling time of ~ 24 – 30 h in general (data not shown).

The Oncogenic Potential of Δ LMP1 EBV-infected B Cells *in Vivo*. Human B cells infected with EBV cause lymphoproliferative malignancies in SCID mice (39). Because LMP1 is known as one of the most important mediators of B-cell transformation, it was quite surprising that we obtained proliferating B cells lacking LMP1 in our *in vitro* experiments. To investigate the oncogenic potential of these cell lines *in vivo*, experiments in SCID mice were done. Two groups of mice were injected i.p. with proliferating B cells derived from the

same human donor, one group with B cells obtained with 2089, the second group with proliferating B cells obtained with Δ LMP1 maxi-EBV. Up to 1×10^8 cells were injected i.p. per animal (Fig. 4). With only one exception, all mice injected with B cells with 2089 wild-type EBV developed malignancies during the first 28 days (Fig. 4). Animal survival correlated with the number of injected cells as expected. Up to 50% of blood cells could be identified as of human origin in some of the mice injected with 2089-infected human B cells, and it was also possible to reestablish the injected B cells from tumors present primarily in liver, kidney, and spleen. The second group of mice injected with $\leq 1 \times 10^8$ B cells obtained with Δ LMP1 EBV stayed healthy for 100 days, and no tumors were detectable (Fig. 4). This result impressively demonstrated the importance of LMP1 for *in vivo* oncogenesis by EBV-positive, growth-transformed B-cell lines.

DISCUSSION

LMP1 was proposed to be essential for cellular transformation of human B cells *in vitro* (35). Our experiments unexpectedly indicate that LMP1 is critical but not mandatory for the process of B-cell proliferation under certain circumstances, such as high virus titers and cocultivation in the presence of human fibroblast feeder cells. We established a panel of EBV mutants in LMP1 based on the maxi-EBV system. With this system in hand, it is possible to generate genetically defined, pure EBV virion stocks with concentrations between 5×10^4 and 1×10^5 infectious particles/ml. For the first time, this approach made it possible to investigate the contribution quantitatively of different LMP1 regions to the process of B-cell proliferation *in vitro*.

Our experiments indicate the existence of different stages of growth-transformed B cells. To clarify these different stages, we would like to introduce a new terminology. Infection of human primary B cells with EBV results in ~ 3 – 10% of the cells proliferating as so-called LCLs (51, 52). In general, such proliferating B cells have been considered to have an indefinite life span (for a review, see Ref. 2). We propose to use the term “phenotype 1” to characterize such cell lines capable of indefinite proliferation in standard growth medium. However, recent data suggest that within a range of 30–150 population doublings, LCLs derived from normal human donor B cells cease to proliferate and die (53). We propose to introduce the term “phenotype 2” to characterize those cells which can be cultivated on plastic in standard growth medium with a finite life span *in vitro*. Only one of four LCLs become immortal after proliferative crisis and continue to proliferate indefinitely to yield phenotype 1 cell lines, a phenomenon which seems to correlate with telomerase expression (53, 54). We now observe a third phenotype of growth-transformed B cells, which is consistent with sustained B-cell proliferation in the presence of human fibroblast feeder cells, only. This remarkable “phenotype 3” clearly differs from growth-transformed phenotype 2 B cells and primary human B cells that are infected with, e.g., an EBV strain lacking EBNA2. The Δ EBNA2 mutant EBV is incapable to induce or maintain B-cell proliferation even when the infected cells were plated on human fibroblast feeder layers under identical conditions (55). This phenotype is not surprising because Δ EBNA2 EBV is essentially null for other critical transforming genes, including EBNA-LP, EBNA3A, EBNA3C, and LMP1, because these are EBNA2 transactivated genes. However, EBV mutants singly deficient for either EBNA3A (48, 56), EBNA3C (Ref. 56) (data to be published), or EBNA-LP (data to be published) are equally impaired. It is interesting to note that previous studies have also shown characteristics of phenotype 3-transformed B cells. Primary B cells infected with an EBV mutant lacking the two Y1 and Y2 exons of EBNA-LP (55) and a particular LMP1 mutant EBV (36) at low multiplicity of infection (37) are similarly dependent on human fibroblast feeder cells for continued proliferation *in vitro*,

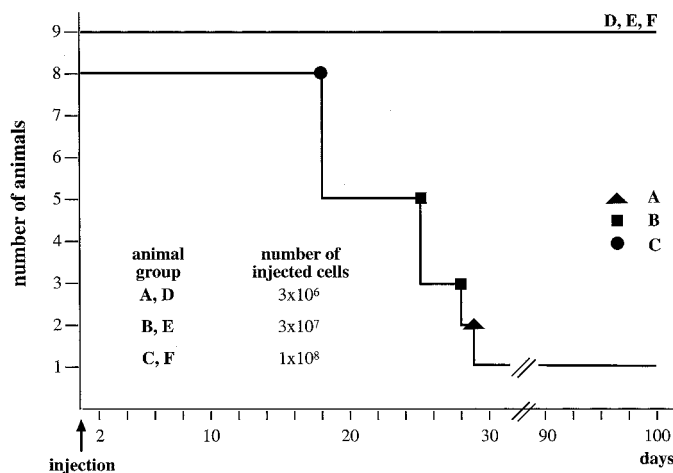


Fig. 4. Survival of SCID mice after injection of 2089 maxi-EBV and Δ LMP1 maxi-EBV growth-transformed B cells. Two B-cell lines, one obtained after infection with 2089 maxi-EBV, the other with Δ LMP1 maxi-EBV from the same donor, were analyzed. With one exception, all nine animals injected i.p. with 2089 maxi-EBV LCL (groups A–C) developed malignancies within a month. There was a strong dependency between time of death and number of cells received. One mouse in group A, which had received 3×10^6 cells, survived. In contrast, all animals injected with Δ LMP1 maxi-EBV B-cell clone (groups D–F) remained healthy without any detectable tumor within the observation period of 100 days. Each group consisted of three mice and received different numbers of cells as indicated. One animal in group A had to be sacrificed because of a spontaneous thymoma.

supporting the characteristics of phenotype 3 growth-transformed B cells by EBV.

Infection of primary human B lymphocytes with EBV in the presence of γ -irradiated human embryo fibroblasts as feeder cells is used as a standard procedure by us *et al.* (36, 37, 44, 48, 49, 55) and does considerably increase the efficiency of clonal outgrowth of proliferating B cells (Ref. 44 and references therein). The effect of this feeder monolayer is mostly mediated by unidentified soluble factors; a direct cell-to-cell contact is not essential to mediate this effect (Ref. 44 and data not shown). Once established, the majority of the proliferating B cells that were generated with the different maxi-EBVs during this study was capable of proliferating in the absence of feeder cells for months. There were three exceptions, however, which turned out to be absolutely dependent on the presence of feeder cells (or conditioned medium; data not shown) for their continued proliferation *in vitro*. B cells derived from maxi-EBV stocks with the mutants Δ C-LMP1, Δ LMP1, and Δ TM-LMP1 required feeder cells for sustained proliferation. Those three EBV mutants also showed a very reduced efficiency of B-cell transformation of 4.1, 1.2, and 0.55%, compared with proliferating B cells established with wild-type maxi-EBV 2089 (Fig. 2). In the presence of irradiated fibroblast feeder cells, B cells infected with these mutant EBVs could be expanded for $\sim$ 40–60 population doublings and maintained routinely for months, indicative of phenotype 3 growth-transformed B cells. To assess the oncogenic potential of a B-cell clone infected with Δ -LMP1 EBV in SCID mice, $>10^9$ cells were needed for the injections (Fig. 4). In the absence of feeder cells, these B-cell clones arrested in the G₀-G₁ phase of the cell cycle and slowly died off after a resting period of 2 weeks (data to be published). This observation indicated that LMP1 directly or indirectly by paracrine effects contributes to cellular proliferation. Obviously, this requirement can be *trans*-complemented by external stimuli, such as feeder cells or conditioned medium. It thus appears that the microenvironment even under *in vitro* cell culture conditions affects proliferation. Feeder cells in an *in vitro* situation might be functionally reminiscent of the stroma of germinal centers or play a similar role as lymph node stroma may do for the initiation of Burkitt's lymphoma.

The fact that some of our EBV mutants support proliferation of B cells is in apparent conflict with previous observations (14, 31, 35). However, we now know that high titer virus stocks are crucial to study EBV mutants that are severely crippled functionally. The efficiency of B-cell transformation is very much reduced with maxi-EBV mutants, such as Δ C-LMP1, Δ TM-LMP1, and Δ LMP1, and $\leq$ 100-fold more viral particles were required to establish a proliferating single cell B-cell clone compared with wild-type EBV. It is likely that those conditions could not be met in previous studies. It is interesting to note that proliferating B-cell lines that needed $\leq$ 100 times more infectious units than wild-type EBV to induce and maintain B-cell proliferation neither retained high copy numbers of EBV genomic DNA (data not shown) nor expressed high levels of viral genes, such as EBNA2 (Fig. 3C) or other genes (data not shown). We favor the explanation that the multiple mutant virions bring information required early in infection to promote the initiation of proliferation because high levels of viral gene expression are not found in those cells that do proliferate after infection with the mutant viruses. Other interpretations of this intriguing finding are possible, too. Very obviously, the clonal transformation of B cells by EBV is affected by mutations within LMP1. Such mutations appear to affect EBV's ability to contribute to the induction and maintenance of cellular proliferation. These events are a necessary prelude to the infected cells ever yielding immortalized progeny.

A number of publications deal with alterations in LMP1's short NH₂ terminus. Such mutations alter the half-life of the LMP1 protein among other effects, such as reduced NF- κ B activation, membrane

localization (15, 26, 27, 57, 58), transformation (35, 47, 59), and cytostatic effects (60). We found that two mutations in this part of LMP1 that alter the putative SH3 domain (61) do not (P12–15–16–19–20A-LMP1) or only marginally (P9–10⁻¹⁵A-LMP1) affect EBV-mediated B-cell growth transformation. Several reports focus on the role of LMP1's COOH-terminal domains in terms of signaling and B-cell transformation. The first 45 aa of the COOH terminus (aa 187–231) known as CTAR1 contains the consensus TRAF-binding motif PxQxT. This region of LMP1 accounts for $\sim$ 25% of LMP1's NF- κ B activity (23, 26, 27, 62) and was proposed to be essential and sufficient for B-cell proliferation when a COOH-terminally truncated LMP1 was investigated (36, 37). A deletion of the CTAR1 motif within the context of full-length LMP1 caused a loss of the immortalizing phenotype (31) in contrast to the discrete mutation of the PxQxT motif in this study, which reduced the efficiency of B-cell transformation to $\sim$ 14%. LCLs established with the maxi-EBV Δ PxQxT-LMP1 could be grown in culture over months in the absence of feeder cells without any noticeable problems.

The proposed JAK3-binding domain in CTAR3 (32) seemed to play no role in the generation of proliferating B-cell clones (18, 63), which was confirmed by our experiments with the maxi-EBV deletion mutant Δ JAK3-LMP1. This finding could mean that the JAK3 interaction domain mapped previously within LMP1's COOH terminus consists of nonconsecutive motifs (Refs. 33 and 34 and data not shown), which might explain the lack of a phenotype of this particular maxi-EBV. Very similar to our findings, JAK3 signaling does not appear to be critical in CD40-mediated B-cell proliferation and other well-known CD40-induced phenotypes (64). The LMP1:CD40 maxi-EBV showed a slightly reduced transformation efficiency, indicating an adequate complementation of LMP1 functions. This phenotypic finding is very much in line with previous reports, suggesting LMP1's mimicry of an activated CD40 molecule (9–11).

Within LMP1's COOH terminus, the region between aa 374 and 384 accounts for $\sim$ 75% of the NF- κ B activity induced by LMP1 (13, 26). This region was shown to bind TRADD (18, 19), which, together with TRAF2 and TRAF6, mediates induction of NF- κ B and the p38 MAPK pathways (18, 19, 22, 28). JNK1/AP1 is also induced via this region, but the signal mediators are less clear (19, 30, 65). In this study, a single point mutation in Y384G-LMP1 maxi-EBV reduced the efficiency of cellular transformation to $\sim$ 10%. We were able to cultivate several cell clones derived from maxi-EBV Y384G-LMP1 stocks from dilutions that met conditions of single-hit kinetics. Although initial conditions in micro-titer plates always included a fibroblast feeder, Y384G-LCL-clones could be easily expanded 6 weeks after infection of the primary cells and cultivated for months without feeder cells in contrast to earlier publications (31, 36, 37).

The Δ TM-LMP1 maxi-EBV disclosed a cellular transformation frequency that was consistently lower than the complete knockout in Δ LMP1 maxi-EBV, suggesting a dominant negative function. Immunoblots demonstrated a steady-state level of the mutated LMP1 protein that was comparable with wild-type LMP1 (Fig. 3A). In contrast, a dramatic overexpression of the Δ C-LMP1 mutant was noticed (Fig. 3B and data not shown). This effect might stem from the deletion of the complete COOH terminus, causing a drastic alteration of the whole molecule (12, 60).

The fact that even a mutant EBV lacking the entire LMP1 gene could induce and maintain proliferating B-cell clones *in vitro* was quite unexpected. Proliferating B-cell clones derived from infection with the Δ LMP1 maxi-EBV mutant were compared with LCLs derived from the same donor established with wild-type maxi-EBV 2089 in the SCID mouse model. Our results indicated, and are in line with data obtained in transgenic mice (6, 7, 10), that LMP1 functions not

only improve the efficiency of B-cell proliferation but are also essential for EBV's oncogenicity in SCID mice.

ACKNOWLEDGMENTS

We thank A. Kieser and B. Sugden for their comments on this manuscript. We also thank J. Middeldorp for the monoclonal antibody OT22CN and E. Kremmer for her gift of other monoclonal antibodies, W. Wackernagel for the plasmid pCP15, and O. Gires for the supply with adenoid samples.

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