Epstein-Barr Virus Recombinant Molecular Genetic Analysis of the LMP1 Amino-Terminal Cytoplasmic Domain Reveals a Probable Structural Role, with No Component Essential for Primary B-Lymphocyte Growth Transformation

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Previous recombinant Epstein-Barr virus molecular genetic experiments with specifically mutated LMP1 genes indicate that LMP1 is essential for primary B-lymphocyte growth transformation and that the amino-terminal cytoplasmic and first transmembrane domains are together an important mediator of transformation. EBV recombinants with specific deletions in the amino-terminal cytoplasmic domain have now been constructed and tested for the ability to growth transform primary B lymphocytes into lymphoblastoid cell lines. Surprisingly, deletion of DNA encoding EHDLER or GPPLSSS from the full LMP1 amino-terminal cytoplasmic domain (MEHDLERGPGRPPRRGPPLSSS) had no discernible effect on primary B-lymphocyte transformation. These two motifs distinguish the LMP1 amino-terminal cytoplasmic domain from other arginine-rich membrane proximal sequences that anchor hydrophobic transmembrane domains. Two deletions which included the ERGPPGPRRPPR motif adversely affected but did not prevent transformation. This arginine- and proline-rich sequence is probably important in anchoring the first transmembrane domain in the plasma membrane, since these mutated LMP1s had altered stability and cell membrane localization. The finding that overlapping deletions of the entire amino-terminal cytoplasmic domain do not ablate transformation is most consistent with a model postulating that the transmembrane and carboxyl-terminal cytoplasmic domains are the likely biochemical effectors of transformation.

Epstein-Barr virus (EBV) can infect and efficiently transform B lymphocytes into immortal lymphoblastoid cell lines (LCLs) (12, 26). The viral genome is usually maintained as a multicopy episome (1, 18), and there is usually no lytic virus infection. Instead, EBV expresses six nuclear proteins (EBNAs 1, 2, 3A, 3B, 3C, and LP), three integral membrane proteins (LMP1, -2A, and -2B), and two small RNAs (EBER1 and -2) (for a review see reference 16). These viral gene products are likely to mediate this latent infection or the accompanying B-lymphocyte growth transformation.

This report describes a recombinant EBV-based molecular genetic analysis of the amino-terminal cytoplasmic domain of LMP1, a gene which is essential to EBV-mediated cell growth transformation (15). LMP1 is an integral membrane protein with a predicted structure consisting of a 24-amino-acid cytoplasmic domain, six membrane-spanning domains separated by short reverse turns, and a 200-amino-acid cytoplasmic domain (10). LMP1 forms a tight patch in the plasma membrane of EBV-infected cells, where it can associate with vimentin (13). Single-gene transfer of LMP1 to EBV-negative Burkitt lymphoma (BL) cell lines recapitulates many of the effects of latent EBV infection in primary B lymphocytes (31). Cells transfected with the LMP1 gene enlarge, grow in clumps, and increase activation marker (CD23 and CD40), adhesion molecule (LFA1, LFA3, and ICAM1), and Bcl-2 expression. LMP1's effects also extend to nonlymphoid cells. Expression of LMP1 in rodent fibroblasts causes cells to grow in medium

containing reduced amounts of serum, to lose contact inhibition, to become anchorage independent, and to produce tumors in nude mice after adoptive transfer (3, 24, 28). Expression of LMP1 under control of a polyomavirus promoter in transgenic mice results in epithelial cell hyperplasia (33). LMP1 also blocks terminal differentiation in a human epithelial cell line (9). These results suggest that LMP1 acts as a dominant, gain-of-function transforming protein by interacting in the plasma membrane with proteins that regulate cell growth.

Several lines of evidence have indicated that the amino terminus and first transmembrane domain of LMP1 are essential to its transforming activity. In rodent fibroblasts or in human BL cells, the last two transmembrane domains and the entire carboxyl terminus of LMP1 are easily expressed as a stable protein which associates with all cytoplasmic membranes without effect on cell growth or gene expression (4, 17, 29, 30). The last five transmembrane domains and the carboxyl-terminal cytoplasmic domain are not sufficient for anchorage-independent growth in BALB/c 3T3 cells or for loss of contact inhibition in Rat-1 cells (4, 24). These two assays yield conflicting results in evaluating the significance of the long carboxyl-terminal cytoplasmic domain (4, 24). More recently, EBV recombinants with a specifically mutated LMP1 gene that encodes the last five transmembrane domains and the carboxyl terminus have been demonstrated to be unable to transform primary B lymphocytes without wild-type (wt) LMP1 being provided in *trans*. The amino-terminally truncated LMP1 was distributed diffusely in the cytoplasm and in focal aggregates along the plasma membrane, and it did not inhibit the effects of wt LMP1 expressed in *trans* (15). These results indicate that the amino-terminal cytoplasmic or first transmembrane do-

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main is essential to wt intracellular localization and transformation. Since the amino-terminal cytoplasmic domain is an unusual sequence which is not found in other proteins and the first transmembrane domain is not remarkably different from other hydrophobic transmembrane domains, we first focused on evaluating the role of the amino-terminal cytoplasmic domain in primary B-lymphocyte growth transformation. We divided this domain into three motifs. The first motif (EHDLE) comprises one weakly basic residue and three strongly acidic residues, the second motif (RGPPGPRRPPR) has four basic arginines and five prolines, and the third motif (GPPLSSS) comprises two prolines and three serines. Since the second motif resembles a transmembrane domain anchor because it is rich in arginine residues, we predicted that deleting one of the other two motifs would remove a critical effector component and adversely affect B-lymphocyte growth transformation. The results were different than anticipated.

MATERIALS AND METHODS

Cells and viruses. The EBV-positive BL cell line P3HR-1 subclone HH514-16 was obtained from George Miller, Yale University. The P3HR-1 cell line is latently infected with a replication-competent but transformation-negative EBV also named P3HR-1 (22). Marker rescue of this P3HR-1 EBV (see below for methods) with cloned EBV DNA that includes the last two exons of EBNA LP and the EBNA 2 exon rescues P3HR-1 for the ability to growth transform primary B cells (7). IB4 is an EBV-transformed LCL generated by infecting cord blood leukocytes with virus from B95-8 marmoset cells (23). BL41 is an EBV-negative BL cell line (5). Primary leukocytes from healthy donors were purified from heparin-treated whole blood by centrifugation on Ficoll-Paque (Pharmacia) density gradients and adsorption to sheep erythrocytes followed by a second round of density gradient centrifugation. Cells were maintained in RPMI 1640 medium supplemented with 10% (vol/vol) fetal bovine serum (Sigma), 10^2 U of penicillin per ml, and 2 μ g of gentamicin per ml at 37°C under a 5 to 7% CO_2 , humidified atmosphere. Nascent LCLs were maintained in the same medium supplemented with 15% fetal bovine serum.

DNA cloning and mutation of the LMP1 gene. B89 is an EBV B95-8 DNA fragment which extends from an *EcoRV* site (map position 165740 [numbering as in reference 2]) across the fused ends of the genome (172281 and 1) and ends at an *Asp* 718 site (3498). B89 includes the LMP1 gene and was cloned into pBC(KS+) (Stratagene). Two single-stranded oligonucleotides (5'-ACATCGATCTGAGGATGGAACACGATCTAGAGAGGGGCCACCGG-3' and 5'-CCTAGGGAGCTCGAGAGGGGCGGTCCGCGAGGGGGCCGTCGCGGGCCCGGTGG-3') were annealed at their 3' ends and mutually primed a Klenow end-fill reaction. The double-stranded product was then inserted into B89 and replaced the wt sequence from a *Bsu36I* site (169476) to an *StuI* site (169397). This introduces unique *ClaI*, *XbaI*, *CpoI*, and *SacI* sites but does not introduce missense mutations in the LMP1 open reading frame. This synthetic wt (S-wt) DNA was further modified at an *EcoRI* site (map position 1) by introducing a 1.8-kbp *BamHI-ClaI* fragment from pBS-HYG, which codes for simian virus 40 (SV40) early region driving expression of hygromycin phosphotransferase (32). Deletion mutations in the LMP1 open reading frame were constructed as follows. S-wt DNA was cut with *CpoI* and *SacI*, mung bean nuclease treated, and ligated to generate clone $\Delta 18-24$. $\Delta 2-7$ was made by cutting S-wt DNA with *ClaI* and *SfiI* and inserting two annealed, *ClaI*-cut oligonucleotides (5'-CCATCGATCTGAGGATGGGCACACC-3' and 5'-GGGCCCATCCTCAGATCGATGG-3').

FIG. 1. Diagram of LMP1 in the plasma membrane demonstrating the 24-amino-acid amino-terminal cytoplasmic domain, six transmembrane domains separated by short reverse turns, and the 200-amino-acid carboxyl-cytoplasmic domain. Codon 44 (methionine [M]), indicated by an arrow, is used by EBV recombinant LMP1MS9 to initiate LMP1 translation (15). Below the diagram, the amino-terminal domain is expanded to reveal the mutated LMP1 expressed by clones S-wt, $\Delta 18-24$, $\Delta 2-7$, $\Delta 6-17$, and $\Delta 6-24$.

$\Delta 6-17$ was made by cutting S-wt DNA with *XbaI* and *CpoI*, Klenow end filling, and ligation. $\Delta 6-24$ was made by cutting S-wt DNA with *XbaI* and *SacI*, treatment with T4 DNA polymerase, and ligation. The nucleotide sequences at sites of manipulation were confirmed by dideoxy sequencing (data not shown), and the predicted amino acid sequences are shown in Fig. 1.

Transfection and infections. Recombinant viruses were generated by transfecting 10^7 P3HR-1 cells in 0.4 ml of medium in a 0.4-cm-gap cuvette (Bio-Rad) with 40 μ g pSVNaeI Z DNA (an expression vector for the BZLF1 immediate-early transactivating protein [8]), 10 μ g of *EcoRI*-A DNA (a cosmid that encodes the EBNA LP and EBNA 2 genes essential for transformation [6]), and 20 μ g of one of the mutated LMP1 DNAs. An electric pulse of 200 V and 960 μ F was delivered, and cells were then diluted in 15 ml of medium. After 5 days at 37°C, cells and supernatant were freeze-thawed twice and passed through a 0.45- μ m-pore-size filter. About 0.5 ml was used to infect 5×10^6 primary lymphocytes. After incubation at 37°C for 2 h, cells were diluted in medium and seeded into 96-well plates (Costar) at 50,000 cells per well (27). Four to six weeks later, LCL colonies were transferred to larger vessels and scored for recombinant virus by PCR analysis (see below).

Recombinant virus was recovered by inducing replication in LCLs by electroporating cells with pSVNaeI Z under the conditions described above except that 240 V was used, and cells were cultured in medium supplemented with 20 ng of phorbol 12-myristate 13-acetate (Life Technologies) per ml (34).

PCR analysis. About 500,000 cells were collected and resuspended in 100 μ l of 10 mM Tris (pH 8.0)–1 mM EDTA. After boiling for 30 min, 10 μ l was amplified for 40 cycles at 94°C for 1 min, 62°C for 1 min, and 72°C for 2 min in a 25- μ l volume of 0.2 mM deoxynucleoside triphosphates, 1.0 μ M LMP1-specific primers (5'-CACGCGTTACTCTGACGTAGCCG-3' and 5'-CTCCAGTCCAGTCACTCATAACG-3'), 0.15 U of *Taq* DNA polymerase (Stratagene), and 1 $\times$ reaction buffer (Stratagene). Amplified DNA products were electrophoretically size separated in 2% (wt/vol) NuSieve agarose–1% (wt/vol)

Seakem ME agarose (FMC Bioproducts)-1× Tris-borate-EDTA gels containing 0.5 µg of ethidium bromide per ml (20). To score for wild-type LMP1 genes in ostensibly recombinant-infected LCLs, wt LMP1 gene-specific primers (5'-CAAGGCCTAGGGAAGAGGAGAG-3' and 5'-GAGGATGGAACACGACCTTGAGA-3' and 5'-TCCTCGAGGGGCGCTCGCG-3') were used.

Western blot (immunoblot) and indirect immunofluorescence analyses. Cells (5×10^4) were denatured by boiling, and proteins were electrophoretically resolved on a denaturing discontinuous 8% polyacrylamide gel. Proteins were electrophoretically transferred to nitrocellulose filters (Schleicher & Schuell), and LMP1 was detected with monoclonal antibody S12, rabbit anti-mouse immunoglobulin (heavy and light chain) [Ig (H+L); Jackson Immunoresearch], and 125 I-labeled protein A (Amersham). Autoradiographic signals were collected on PhosphorImager screens (Molecular Dynamics), and data were analyzed with the manufacturer's computer and Image-Quant software and with Molecular Dynamics Image and Computer Associates Cricket Draw III software on an Apple Macintosh Quadra. To stain for LMP1 in situ, LCLs were fixed with 50% (vol/vol) methanol-50% (vol/vol) acetone on slides (Polysciences) and probed with monoclonal antibody S12 and fluorescein-conjugated goat anti-mouse Ig (H+L) secondary antibody (Jackson Immunoresearch). To score for virus replication in LCLs induced by pSVNaeI Z electroporation and phorbol ester treatment (see above), fixed cells were stained for late lytic infection antigen gp110 with monoclonal antibody L2 (A gift from Gary Pearson, Georgetown University) and fluorescein-conjugated goat anti-mouse Ig (H+L) secondary antibody.

Southern blot analyses. Cells (10^7) were sedimented, lysed in 3 ml of 50 mM Tris (pH 8)-10 mM EDTA-and 1% sodium dodecyl sulfate (SDS), and digested with 0.15 mg of proteinase K per ml at 55°C for 1 h. Samples were phenol-chloroform extracted, ethanol precipitated, resuspended in water, and endonuclease digested. Ten micrograms of DNA was electrophoretically separated in 0.8% (wt/vol) agarose-1× Tris-borate-EDTA gels containing 0.5 µg of ethidium bromide per ml (20), pressure blotted (Stratagene) to nylon filters (MSI Mag-nagraph), and probed with DNA labeled with [32 P]dCTP by a random-primer-based method (Multiprime DNA labeling system; Amersham). After stringent washing ($0.1 \times$ SSC [$1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate]-0.5% SDS, 68°C, 1 h) (20), autoradiographic signals were collected on PhosphorImager screens and analyzed by computer software as described for Western immunoblots.

RESULTS

Mutation of the LMP1 gene. A cloned EBV DNA fragment was mutated by replacing the DNA segment which encodes the amino terminus of LMP1 with a double-stranded oligonucleotide that includes new restriction sites that are unique to the cloned plasmid DNA but does not alter the protein sequence. Deletions of specific codons were then made by cleavage at the unique sites, modification of the DNA ends or insertion of a mutated double-stranded oligonucleotide, and ligation of the plasmid DNA. The specific manipulations for each mutation are described in Materials and Methods. Each mutation was sequenced at the site of modification. The predicted primary amino acid sequences encoded by the mutated LMP1 genes are shown in Fig. 1. S-wt is the plasmid that has the new unique restriction endonuclease sites but encodes wt LMP1. Wild-type LMP1 has the amino-terminal cytoplasmic sequence MEHD LERGPPGPRRPPRGPPPLSSS before the first hydrophobic

transmembrane domain. $\Delta 18-24$ is deleted for amino acids 18 to 24. $\Delta 2-7$ is deleted for amino acids 2 to 7. $\Delta 6-17$ is deleted for amino acids 6 to 17. $\Delta 6-24$ is deleted for amino acids 6 to 24.

EBV recombinants with specifically mutated LMP1 genes. To generate EBV recombinants with altered LMP1 genes, P3HR-1 cells, which are latently infected with a transformation-incompetent EBV deleted for EBNA LP and EBNA 2, were electroporated with mutated LMP1 DNA, *EcoRI*-A (a DNA fragment that includes the DNA for which P3HR-1 is deleted and can therefore rescue P3HR-1 virus for the ability to growth transform B cells [7]), and pSVNaeI Z (an expression vector for the BZLF1 immediate-early transactivator of lytic EBV infection [8]). In an electroporated P3HR-1 cell, replication of the endogenous P3HR-1 EBV is induced by BZLF1 protein, and replicating viral DNA may incorporate the cotransfected EBV DNA fragments by homologous recombination. Fresh primary B cells were infected with the virus progeny, seeded into 96-well microtiter plates, and cultured in vitro. Since the parental P3HR-1 EBV is incapable of transforming cells, all LCLs that arise 4 to 8 weeks after infection are latently infected with a recombinant virus that has been reconstituted for the EBNA LP and EBNA 2 genes (7). Also, since replicating EBV genomes that have recombined with one EBV DNA fragment frequently recombine with a second cotransfected EBV DNA fragment (27), some of the LCLs are likely to be infected with a recombinant which has also acquired the mutated LMP1 gene and lost the wt gene. Parental P3HR-1 EBV is produced in 10^5 -fold excess over transforming recombinants so that many of the LCLs are initially coinfecting with P3HR-1 EBV, which can provide wt LMP1 in *trans*. Thus, as long as the mutation does not result in a dominant negative phenotype, LCLs that are coinfecting with mutated LMP1 EBV recombinants and parental P3HR-1 EBV should arise.

The first-generation LCLs were scored for their LMP1 genotype by a PCR-based method with oligonucleotide primers that result in the amplification of LMP1 DNA inclusive of each of the mutation sites. The mutated DNA could be distinguished from the wt DNA by the presence of new restriction sites or by the smaller size of the amplified DNA in those experiments in which the mutation was a deletion. In most experiments, as many as 20% of the LCLs were infected with an EBV recombinant that had mutated LMP1 DNA. These LCLs also had wt LMP1 DNA from P3HR-1 (data not shown).

EBV recombinants with specifically mutated LMP1 genes can transform primary B lymphocytes. To establish the growth-transforming potential of each LMP1 mutation, replication of the mutated recombinant and P3RH1 EBV genomes was induced in the coinfecting LCLs by electroporating the cells with pSVNaeI Z DNA and by cultivating cells in medium supplemented with phorbol ester. Fresh human B lymphocytes were infected with filtered (0.45-µm-pore-size filter) progeny virus, and LCLs that arose were scored for LMP1 genotype by PCR. Since the amplified product of the S-wt LMP1 DNA is identical in size to wt LMP1 DNA and the $\Delta 2-7$ and $\Delta 18-24$ amplified products are only slightly smaller than wt DNA, these PCR products were cut with a restriction enzyme to distinguish mutated and wt LMP1 DNA. The amplified products from LCLs infected with a $\Delta 6-17$ or $\Delta 6-24$ EBV recombinant could be distinguished from wt DNA by size. For each mutation, some LCLs which were infected with only an EBV recombinant that had an EBV genome with a mutated LMP1 gene arose. Examples of the PCR analysis of such LCLs are shown in Fig. 2A. An S-wt-infected LCL amplified DNA (lane

FIG. 2. PCR analyses of mutated LMP1 recombinant EBV-infected LCLs for LMP1 DNA. (A) DNA from 5×10^4 cells or 100 μ g of plasmid DNA was amplified with primers (5'-CACGCGTTACTCTGACGTAGCCG-3' and 5'-CTCCAGTCCAGTCACTCATAACG-3') that bracket the mutated amino-terminal region. Amplified DNA was cut with *Cla*I (lanes 1 to 4), with *Sac*I (lanes 5 and 6), or with both enzymes (lane 11) and resolved in agarose gels containing ethidium bromide. Lanes: 1, LCL S-wt; 2, S-wt plasmid DNA; 3, LCL Δ 18-24; 4, Δ 18-24 plasmid DNA; 5, LCL Δ 2-7; 6, Δ 2-7 plasmid DNA; 7, LCL Δ 6-17; 8, Δ 6-17 plasmid DNA; 9, LCL Δ 6-24; 10, Δ 6-24 plasmid DNA; 11, P3HR1 cells. Molecular markers in base pairs are indicated at the left. (B) PCR analysis for wt LMP1 DNA with primers 5'-CAAGGCCTAGGGAAGAGGAGAG-3' and 5'-GAGGATGGAACACGACCTTGAGA-3'. Lanes 1 to 6 contain 5×10^4 IB4 cells (one EBV copy per cell) serially 10-fold diluted with 5×10^4 EBV-negative BL41 cells and then PCR analyzed. The endpoint dilution in lane 4 is the 10^{-3} dilution for a sensitivity of 50 copies per 5×10^4 cells. No wt LMP1 DNA was detected in 5×10^4 LCL Δ 18-24 (lane 8), LCL Δ 2-7 (lane 9), or EBV-negative BL41 cells (lane 7). Molecular markers in base pairs are indicated on the left. (C) PCR analysis for wt LMP1 DNA with primers 5'-TCCTCGAGGGGCGCTCGC-3' and 5'-CACGCGTTACTCTGACGTAGCCG-3'. Lanes 1 to 6 contain 5×10^4 IB4 cells (one EBV copy per cell) serially 10-fold diluted with 5×10^4 EBV-negative BL41 cells and then PCR analyzed. The endpoint dilution is between lanes 3 and 4, or the 10^{-2} to 10^{-3} dilutions for a sensitivity of 500 to 50 copies per 5×10^4 cells. No wt LMP1 DNA was detected in 5×10^4 LCL Δ 6-17 (lane 8), LCL Δ 6-24 (lane 9), or EBV-negative BL41 cells (lane 7). Molecular markers in base pairs are indicated on the left.

1) is completely cut at a *Cla*I site similar to the mutated plasmid DNA (lane 2), while the parental P3HR-1 EBV DNA lacks this site (lane 11). A Δ 18-24-infected LCL amplified DNA is completely cut with *Cla*I (lane 3), as is the product amplified from the plasmid DNA that was used to make this recombinant (lane 4). A Δ 2-7-infected LCL amplified DNA is completely cut with *Sac*I (lane 5), as is the product from the plasmid DNA (lane 6). The amplified DNA from LCLs infected with Δ 6-17 (lane 7) and Δ 6-24 (lane 9) recombinants is identical in size to products from the respective plasmid DNA (lanes 8 and 10). Both products are significantly smaller than the product from wt P3HR-1 LMP1 DNA (lane 11).

LCLs infected with mutated LMP1 EBV recombinants lack wt DNA. To assess the possibility of low-level wt LMP1 DNA in the LCLs which appeared to have only a mutated LMP1 EBV recombinant, PCR primers specific for wt LMP1 DNA were used in an attempt to detect far fewer than one wt LMP1

FIG. 3. Southern blot analysis of mutated LMP1 recombinant EBV-infected LCLs. (A) Genetic and restriction maps of EBV. The top line indicates the EBV genome with unique regions 1 to 5 (U1 to U5), internal repeat regions (IR; marked with cross bars), and terminal repeat regions (TR; marked with cross bars). Below is an expanded segment of the DNA comprising the LMP1 gene. The LMP1 transcript (arrow), exons (boxes), and mutated region (*) that encodes new *Cla*I or *Sac*I sites are diagrammed. The *Mlu*I 2.4 Southern blot probe appears on the bottom line. (B) Southern blot of size-separated DNA extracted from LCLs and probed with *Mlu*I 2.4. DNA was cut with *Mlu*I and *Cla*I except for the sample in lane 4, which was cut with *Mlu*I and *Sac*I. Lanes: 1, BL41 cells; 2, LCL S-wt; 3, LCL Δ 18-24; 4, LCL Δ 2-7; 5, LCL Δ 6-17; 6, LCL Δ 6-24; 7, P3HR1 cells. Molecular weight markers in kilobase pairs are indicated on the left. Autoradiographic signals were collected on PhosphorImager screens (Molecular Dynamics), and data were analyzed with the manufacturer's computer and ImageQuant software and with Molecular Dynamics Image and Computer Associates Cricket Draw III software on an Apple Macintosh Quadra.

gene per cell. The results are shown in Fig. 2B. Primers which can detect wt LMP1 DNA with a level of sensitivity of 50 wt copies per 50,000 cells (lane 4) fail to amplify wt LMP1 DNA from LCLs infected with a Δ 18-24 (lane 8) or Δ 2-7 (lane 9) EBV recombinant. In lanes 4, 5, 6, 8, and 9, a PCR-amplified DNA migrating with the 134-bp DNA marker but not specific for LMP1 DNA can also be seen. Another set of PCR primers which can detect between 50 to 500 wt LMP1 copies per 50,000 cells (Fig. 2C, lanes 3 and 4) fails to detect wt LMP1 DNA in LCLs infected with a Δ 6-17 (lane 8) or Δ 6-24 (lane 9) EBV recombinant. These data demonstrate that the Δ 2-7, Δ 6-24, Δ 6-17, and Δ 18-24 mutated LMP1 EBV recombinants can transform primary B lymphocytes and not do not require wt LMP1 DNA.

Gene organization in mutated LMP1 recombinant EBV-infected LCLs. The LMP1 genes of the specifically mutated EBV recombinants were examined by Southern blot analysis. DNA from LCLs was digested with *Mlu*I and *Cla*I or *Mlu*I and *Sac*I, electrophoretically size separated in agarose gels, blotted to filters, and probed with a 2.4-kbp *Mlu*I (*Mlu*I 2.4) DNA fragment that encompasses the LMP1 open reading frame (Fig. 3A). The S-wt, Δ 18-24, Δ 6-17, and Δ 6-24 LMP1 muta-

FIG. 4. Immunoblot analysis of mutated LMP1 recombinant EBV-infected LCLs. About 5×10^4 cells were lysed and resolved in denaturing polyacrylamide gels. After Western transfer to nitrocellulose filters, LMP1 was detected with monoclonal antibody S12 and radioiodinated protein A. Lanes: 1, BL41 cells; 2, IB4 cells; 3, LCL S-wt; 4, LCL $\Delta 18-24$; 5, LCL $\Delta 2-7$; 6, LCL $\Delta 6-17$; 7, LCL $\Delta 6-24$. Molecular weight markers in kilodaltons and positions of LMP1 and D1LMP1 are indicated on the left. Autoradiographic signals were collected on PhosphorImager screens (Molecular Dynamics), and data were analyzed with the manufacturer's computer and ImageQuant software and with Molecular Dynamics Image and Computer Associates Cricket Draw III software on an Apple Macintosh Quadra.

tions have a new *Cla*I site, the $\Delta 2-7$ LMP1 mutation has a new *Sac*I site, and wt LMP1 EBV DNA has neither *Cla*I nor *Sac*I sites within the *Mlu*I 2.4 fragment. The blot in Fig. 3B, lane 7, shows that the probe hybridized to a 2.4-kbp wt LMP1 DNA fragment which was not cut by *Cla*I or *Sac*I and to a slightly smaller fragment from LCLs infected with an S-wt (lane 2), $\Delta 18-24$ (lane 3), $\Delta 2-7$ (lane 4), $\Delta 6-17$ (lane 5), or $\Delta 6-24$ (lane 6) EBV recombinant. The smaller fragment is identical in size to that expected from cleavage at *Cla*I or *Sac*I sites in the mutated DNA. The other part of the 2.4-kbp fragment of the mutated DNAs that was cleaved by *Cla*I or *Sac*I and hybridizes to the probe is about 110 bp and ran off the gel. The blot also confirms that there is no 2.4-kbp wt LMP1 DNA in these LCLs, as was shown with much greater sensitivity by the PCR analyses described before.

LMP1 in EBV recombinant-infected LCLs. LMP1 expression was evaluated by immunoblotting with monoclonal antibody S12. Figure 4 demonstrates that all of the EBV recombinants express LMP1 cross-reactive proteins. By PhosphorImager analysis, LCLs infected with S-wt (lane 3), $\Delta 18-24$ (lane 4), and $\Delta 2-7$ (lane 5) EBV recombinants expressed about the same amount of LMP1, while LCLs infected with the $\Delta 6-17$ (lane 6) or $\Delta 6-24$ (lane 7) recombinant expressed two or three times the steady-state levels of LMP1. Higher-level expression of amino-terminally truncated, codon 44-initiated LMP1 was previously observed with EBV recombinants and may reflect enhanced protein stability (15). The LCLs infected with the S-wt (lane 3), $\Delta 6-17$ (lane 6), and $\Delta 6-24$ (lane 7) EBV recombinants shown in Fig. 4 also express a small amount of D1LMP1 which is associated with lytic EBV infection. This is probably due to the presence of SV40 early-region sequences in these EBV recombinants. The cloned DNAs from which all of the recombinants were constructed included an SV40-hygromycin phosphotransferase gene at a site separated from the LMP1 promoter by the terminal repeats (Fig. 3A). PCR analyses reveal that these LCLs are infected with EBV recom-

FIG. 5. Immunofluorescent staining of methanol-acetone-fixed LCLs with monoclonal antibody S12 and fluorescein-conjugated secondary antibody. (A) IB4 cells demonstrating that LMP1 is localized to the plasma membrane aggregated in a patch; (B) LCL S-wt; (C) LCL $\Delta 18-24$; (D) LCL $\Delta 2-7$; (E) LCL $\Delta 6-17$; (F) LCL $\Delta 6-24$. LMP1 in LCLs $\Delta 6-17$ and $\Delta 6-24$ is more abundant and distributed diffusely in the cytoplasm and in focal aggregates along the plasma membrane.

binants that had incorporated the SV40-hygromycin phosphotransferase gene, while LCLs infected with the $\Delta 2-7$ or $\Delta 18-24$ recombinant did not incorporate this gene (data not shown). Previously, insertion of the SV40-hygromycin phosphotransferase gene at this site has been associated with expression of D1LMP1 without expression of other lytic infection genes (19, 21). LMP1 in LCLs infected with the $\Delta 6-17$ (lane 6) or $\Delta 6-24$ (lane 7) EBV recombinant is slightly smaller than wt LMP1 (lane 2) as a result of the deletion of 12 or 19 codons, respectively. LMP1 in LCLs infected with an S-wt (lane 3), $\Delta 18-24$ (lane 4), or $\Delta 2-7$ (lane 5) EBV recombinant is indistinguishable in size from wt LMP1 (lane 2).

Indirect immunofluorescent staining of fixed LCLs with monoclonal antibody S12 revealed that wt LMP1 was tightly patched and capped in the plasma membrane. LMP1 abundance and localization in LCLs infected with an S-wt (Fig. 5B), $\Delta 18-24$ (Fig. 5C), or $\Delta 2-7$ (Fig. 5D) EBV recombinant were

TABLE 1. Transforming efficiency of recombinant EBV^a

EBV recombinant	% of cells staining positive for gp110	Transforming units/ml	
		Exp 1	Exp 2
S-wt	18.7	9.5×10^2	1.6×10^3
$\Delta 18-24$	15.5	1.1×10^4	1.5×10^4
$\Delta 2-7$	28.4	2.1×10^4	8.0×10^4
$\Delta 6-17$	14.5	1.1×10^2	1.5×10^2
$\Delta 6-24$	10.5	1.5×10^1	2.6×10^0

^a EBV recombinant-infected LCLs were electroporated with pSVNaeI Z, a DNA expression plasmid for the EBV immediate-early transactivator, and treated with phorbol ester to induce lytic infection. After 5 days, a portion of cells was fixed, stained with monoclonal antibody L2 against late lytic infection glycoprotein gp110 and immunofluorescent secondary antibody, and examined by fluorescence microscopy to score for virus replication. Cells and media were frozen and thawed to release virus. Cell debris was pelleted, and the crude virus preparation was passed through a 0.45- μ m-pore-size filter. This virus preparation was serially diluted and used to infect primary B lymphocytes in a clonal transformation assay. The number of LCLs growing out after 6 weeks in culture was used to calculate the transforming units per milliliter. A portion of the virus preparation was boiled, serial twofold diluted, and PCR analyzed for LMP1 genes with primers 5'-CACGCGTTACTCTGACGTAGCCG-3' and 5'-CTCCAGTCCAGTCACTCATAACG-3' as described in Materials and Methods. The viral DNA titer in cell supernatants, (reciprocal of the maximum dilution at which an LMP1-specific, PCR-amplified DNA product is detectable) was 16 in all cases.

not remarkably different from those of wt EBV-infected IB4 cells (Fig. 5A). LMP1 in LCLs infected with $\Delta 6-17$ (Fig. 5E) and $\Delta 6-24$ (Fig. 5F) EBV recombinants was more abundant in many cells, consistent with immunoblot analysis. In these two LCLs, LMP1 was distributed diffusely in the cytoplasm and in focal aggregates along the plasma membrane. This result is similar to that previously observed for codon 44-initiated LMP1, which is deleted for the amino-terminal cytoplasmic and first transmembrane domains (Fig. 1 and reference 15).

Transformation of primary B lymphocytes with EBV recombinants carrying specific LMP1 mutations. EBV replication was induced in the LCLs shown in Fig. 2 to 5, which are infected with EBV recombinants that have specifically mutated LMP1 genes. Filtered (0.45- μ m-pore-size filter) supernatants from cells induced to similar levels of virus replication were used to infect and potentially transform primary B lymphocytes. The newly infected lymphocytes were plated under conditions in which the relative transforming efficiency of recombinant virus could be assessed by endpoint dilution in a clonal transformation assay (Table 1). EBV from LCLs infected with a $\Delta 18-24$ or $\Delta 2-7$ EBV recombinant readily transformed primary B lymphocytes, while EBV from LCLs infected with a $\Delta 6-17$ or $\Delta 6-24$ EBV recombinant was less active in transformation. The transforming virus titer from LCLs infected with a $\Delta 18-24$ or $\Delta 2-7$ EBV recombinant was 1.1×10^4 to 8.0×10^4 transforming units per ml, while the titer from LCLs infected with a $\Delta 6-17$ or $\Delta 6-24$ EBV recombinant was about 3 to 150 transforming units per ml. The difference in transforming virus titers is in striking contrast to the similar levels of virus replication. After inducing virus replication, cells were stained by an indirect immunofluorescent antibody directed against late lytic antigen gp110. As shown in Table 1, 10 to 30% of the cells expressed late lytic antigen gp110, indicating that cells were induced to replicate virus similarly. Also, the amount of virus in the supernatants used to infect primary B lymphocytes was compared by titrating viral DNA with a PCR-based method. The relative titers of viral DNA in the supernatants were identical (Table 1). Thus, the $\Delta 6-17$ and $\Delta 6-24$ EBV recombinants are deficient in the initiation of growth transformation.

Finally, the outgrowth of $\Delta 6-17$ and $\Delta 6-24$ recombinant-infected LCLs was retarded relative to that of S-wt, $\Delta 18-24$, or $\Delta 2-7$ recombinant EBV-infected LCLs. While the S-wt, $\Delta 18-24$, or $\Delta 2-7$ EBV recombinant-infected LCLs were mostly visible at 3 weeks after infection as is usually observed for wt EBV, the $\Delta 6-17$ or $\Delta 6-24$ recombinant EBV-infected LCLs were mostly not evident until 4 to 6 weeks after infection.

DISCUSSION

These experiments analyze the contribution of specific amino acid residues to the role of LMP1 in EBV-mediated B-lymphocyte growth transformation. Previous recombinant EBV molecular genetic experiments with specifically mutated LMP1 genes demonstrated that LMP1 is essential for LCL outgrowth (15). In those experiments, an amino-terminally truncated LMP1 which begins with codon 44 at the end of the first transmembrane domain (Fig. 1) could not transform primary B lymphocytes into LCLs. The mutated protein was stable and accumulated diffusely in the cytoplasmic and in focal aggregates along the plasma membranes at higher than wt LMP1 abundance. The amino-terminally truncated protein did not inhibit the transforming effects of wt LMP1 encoded from a *trans*-complementing defective viral genome. Despite high-level expression of both mutated and wt LMP1, the coinfecting LCLs were indistinguishable in growth from those infected with wt EBV recombinants. In addition, both mutated and wt genes were stably maintained in many LCLs. Until the current experiments, the simplest interpretation of the essentiality of the amino-terminal 43 amino acids and the lack of a dominant negative effect of stable abundant expression of the truncated protein was that the truncation removes the major effector domain of LMP1 for B-cell growth transformation and the residual five transmembrane and carboxyl-terminal cytoplasmic domains do not themselves interact with downstream effectors. Since the obvious unique attribute of the first 43 amino acids is the amino-terminal cytoplasmic domain, this domain was likely to be the putative effector component. Other interpretations of the data were considered and probably now pertain.

The experiments reported here indicate that there is unlikely to be an essential interaction mediated by the amino-terminal cytoplasmic domains. Any part of the amino-terminal cytoplasmic domain could be deleted without destroying the ability of the mutated LMP1 to participate in primary B-lymphocyte growth transformation. Thus, the amino terminus is not a key effector of transforming activity. In fact, deletion of the most unusual components of the amino-terminal cytoplasmic domain, i.e., EHDLER or GPPLSSS (amino acids 2 to 7 or 18 to 24, respectively), had no negative effect. These mutated LMP1s were expressed at near normal levels and associated with the plasma membrane in a tight patch similarly to wt LMP1. Cells transformed by these specifically mutated LMP1 recombinant EBV were also similar to wt EBV in growth. The residual amino-terminal cytoplasmic sequence, RGPPGPRRPPR (amino acids 7 to 17), is similar to other anchoring domains for hydrophobic membrane insertion sequences such as the transmembrane domains of LMP1. Not surprisingly, deletion of this domain resulted in focal aggregation along the plasma membrane of these stable and relatively abundant LMP1 cross-reactive proteins. Transformation was inhibited and the transformed cells grew less well, but LCLs which were infected with the mutated LMP1 EBV recombinants and lacked wt LMP1 could be obtained. Once established, these LCLs grew as well as wt EBV-transformed LCLs.

The current experiments therefore fundamentally alter our

working model for LMP1 effector domains. These and the previous experiments are now most consistent with the model that the principal function of the amino-terminal cytoplasmic domain is to tether the amino terminus of the first transmembrane domain to the cytoplasm. Presumably this permits the six hydrophobic transmembrane domains and the carboxyl terminus to noncovalently associate in the plasma membrane with other LMP1 and cell molecules to permit the essential transforming interaction to be mediated by the transmembrane domains or carboxyl-terminal cytoplasmic domain.

In focusing attention on the transmembrane domains and carboxyl terminus as effectors of the transformation phenotype, LMP1 could now be viewed as possibly being similar to bovine papillomavirus E5 protein. E5 has a hydrophobic transmembrane domain and a single cytoplasmic domain. E5 interacts with plasma membrane-associated growth factor receptors through its transmembrane and cytoplasmic carboxyl-terminal domains (6, 11, 14, 25).

Little is as yet known about the relative roles of the transmembrane and carboxyl-terminal cytoplasmic domains in any assay of LMP1's activating or transforming effects. Expression of a mutated LMP1 gene encoding a deletion of the third and fourth transmembrane domains in BL cells resulted in altered cytoplasmic membrane localization, loss of the normal effect in inducing cell clumping, and the loss of some but not all activating effects (30). Expression of mutated LMP1 genes encoding deletions of the carboxyl-terminal cytoplasmic domain in BALB/c 3T3 cells resulted in a wt phenotype with regard to induction of anchorage-independent growth, while expression of similar mutations in Rat-1 cells resulted in a null phenotype with regard to loss of contact inhibition (4, 24). Thus, mutations in the transmembrane and carboxyl-terminal cytoplasmic domains must now be investigated by using an EBV recombinant molecular genetic approach. One advantage of this latter approach is that LCL outgrowth is a definitive endpoint. Further definition of the critical transforming domains of LMP1 is important for elucidating the mechanism by which LMP1 contributes to EBV-mediated B-lymphocyte growth transformation.

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