The Last Seven Transmembrane and Carboxy-Terminal Cytoplasmic Domains of Epstein-Barr Virus Latent Membrane Protein 2 (LMP2) Are Dispensable for Lymphocyte Infection and Growth Transformation In Vitro

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Specifically mutated Epstein-Barr virus (EBV) recombinants which truncate latent membrane protein 2A (LMP2A) and LMP2B after 260 of 497 amino acids and after 141 of 378 amino acids, respectively, were constructed. Despite truncation before the last seven transmembrane domains and the carboxy terminus, the mutant recombinants were not altered in initiation of primary B-lymphocyte infection or growth transformation, in expression of nuclear protein 1 or 2 or LMP1, or in induction of lytic EBV replication. Cells transformed by mutant virus recombinants were not different from wild-type virus transformants in initial or long-term outgrowth, sensitivity to limiting cell dilution, serum requirement, or clonogenic growth in soft agar. Together with similar analyses of a mutation stopping translation of the LMP2A amino-terminal cytoplasmic domain, these results indicate that LMP2 is not required for primary B-lymphocyte infection in vitro.

Epstein-Barr virus (EBV) is a human herpesvirus which is the causative agent of infectious mononucleosis in normal adolescents and lymphoproliferative disease in immunocompromised patients and in marmosets (13, 22). EBV is also etiologically associated with African Burkitt's lymphoma, nasopharyngeal carcinoma, some forms of Hodgkin's disease, and some unusual malignancies of epithelial or lymphatic lineages (2, 7, 9, 10, 12, 13, 22, 26, 32, 33, 40). The EBV host range in vitro is restricted to B lymphocytes, which become latently infected and are thereby transformed to perpetual proliferation. These latently infected B lymphocytes will grow as lymphoblastoid cell lines (LCLs). Early-passage LCLs are sensitive to cell and serum concentration, will clone with low efficiency in soft agarose (28, 34), and are tumorigenic in SCID mice, marmosets, and immunocompromised humans (15, 29, 31, 38, 42). They express a restricted repertoire of EBV gene products including two small RNAs, six nuclear proteins (EBNAs), and two integral membrane proteins (LMP1 and LMP2) (13). The EBV genes expressed in latent infection are being intensely studied since they are likely mediators of B-lymphocyte growth transformation.

The experiments reported here investigate the role of LMP2 proteins in primary B-lymphocyte infection in vitro. The LMP2 gene encodes two proteins, LMP2A and LMP2B, whose mRNAs are transcribed from two distinct promoters (14, 30). The mRNAs have different 5' exons followed by eight common exons (14, 30). The LMP2A primary amino acid sequence includes 119 hydrophilic amino acids at the amino terminus, 12 hydrophobic predicted transmembrane domains interrupted by short hydrophilic domains, and a 27-amino-acid carboxy-terminal domain. The amino and carboxy termini are likely to be in the cytoplasm. LMP2B

initiates at a methionine 120 amino acids into LMP2A and lacks the entire amino-terminal domain. LMP2A and LMP2B mRNAs are expressed in latently infected B lymphocytes and in tumor biopsy specimens from patients with nasopharyngeal carcinoma (3, 6). LMP2A is implicated in transmembrane signal transduction because of its association with LMP1 and with important B-lymphocyte tyrosine kinases (4, 5, 16, 41).

In a previous study we demonstrated the feasibility of creating EBV recombinants with specific LMP2 mutations (18). In this previous study, a stop codon was inserted into codon 20 of LMP2A, and this was predicted to stop translation of LMP2A after amino acid 19. The next methionine codon is codon 120, which initiates translation of LMP2B. Despite the lack of LMP2A expression, the mutant EBV recombinant was fully able to initiate latent growth-transforming infection in B lymphocytes and to replicate following induction of the virus lytic cycle. These results indicate that the amino-terminal cytoplasmic domain of LMP2A is dispensable for in vitro B-lymphocyte infection (18). This was particularly surprising since the amino terminus and first two transmembrane domains are sufficient for tyrosine kinase interaction (16), and it is unlikely that the transmembrane domains are the basis for this interaction.

To further evaluate the role of LMP2A and LMP2B in EBV infection, we created EBV recombinants mutated in both LMP2A and LMP2B by inserting a nonsense codon into the 5th of the 12 transmembrane domains. The insertion is predicted to terminate translation of the LMP2A 497-amino-acid sequence at codon 260 and of the LMP2B 378-amino-acid sequence at codon 141. The mutant recombinant virus was then tested in primary B-lymphocyte infection and growth transformation. Resulting LCLs infected with the LMP2A and LMP2B mutant EBV recombinants were also tested in cell growth assays.

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MATERIALS AND METHODS

Cells. BJAB is an EBV-negative B-lymphoma (BL) cell line (21). The HH514-16 subclone of the EBV-infected P3HR1 (P3JHR1) Burkitt's lymphoma cell line (a gift of G. Miller) has EBV genomes which are deleted for a segment which encodes EBNA2 and the last two exons of EBNALP (11). B95-8 is an EBV-infected marmoset cell line that is permissive for viral replication (23, 24). LCL1 and LCL2 are B95-8 EBV-transformed fetal (LCL1) or adult (LCL2) LCLs and are not permissive for viral replication. Primary human B lymphocytes were prepared from peripheral blood, infected with EBV, and plated into microwells for clonal transformation (20). Newly transformed cell lines were maintained in RPMI 1640 medium supplemented with 15% inactivated fetal bovine serum and gentamicin (2 µg/ml). All other cell lines were maintained in RPMI 1640 medium supplemented with 10% inactivated fetal bovine serum and gentamicin (2 µg/ml).

Plasmids and cosmid construction. pRL60 contains a *SacI* (B95-8 bp 163473)-to-*KpnI* (B95-8 bp 3502) fragment from B95-8 EBV DNA. A stop codon was introduced in codon 260 of LMP2A and codon 141 of LMP2B (pLMP2A260S) by cloning an *XbaI* linker (CTAGTCTAGACTAG) containing stop codons in all three frames into the unique *SacI* site of pRL60. The *SacI* site was filled in with the Klenow fragment of DNA polymerase 1. pSVNaeZ was used to induce lytic EBV replication (35). *EcoRI*-A was used to rescue the EBNA2 and EBNALP mutation contained in the P3HR1 cell line (37).

PCR analysis and sequencing of PCR-amplified DNA. Four LMP2A-LMP2B oligonucleotides (see Fig. 1, line 3, for their location and orientation) were synthesized on an Applied Biosystems synthesizer and purified. Oligonucleotides 5'LMP2A/B (ATGCTTGTGCTCCTGATACT) and 5'WTLMP2A/B (ACTTGTCCTCATCGTCGACG) correspond to bases 547 to 566 and bases 630 to 649 of the B95-8 sequence, respectively. Oligonucleotides 3'LMP2A/B (GAGCTGTA ACTGTGGTTTCC) and 3'WTLMP2A/B are complementary to bases 630 to 649 and bases 797 to 816 of the B95-8 sequence, respectively. Cell DNA was prepared for polymerase chain reaction (PCR), amplified, and analyzed by electrophoresis (18, 35).

The nucleotide sequence of the mutation site in recombinant virus-transformed LCLs was verified after PCR amplification with a kit purchased from Pharmacia.

Additional techniques and reagents. Cell transfection by electroporation, the induction of lytic EBV infection, primary human B-lymphocyte infection, and assays of EBV-infected cell growth were performed as previously described (18, 34). Positive-pressure Southern transfer (Stratagene) onto GeneScreen Plus (Dupont), hybridization to cloned ³²P-labeled EBV DNA, autoradiography, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotting were also as previously described (1, 17, 39). Polyclonal rabbit antibody against LMP2A (17), S12 monoclonal antibody directed against LMP1 (19), and polyvalent human EBV immune sera directed against EBNA2 were used in immunoblots.

RESULTS

Construction of LMP2A and LMP2B mutant EBV recombinants. The strategy for deriving an LMP2A- and LMP2B-mutated EBV recombinant by using second-site homologous recombination in the EBNA2- and EBNA2-deleted P3HR1

cell line has been detailed previously (18, 36, 37). Briefly, a nonsense linker was inserted into a unique *SacI* site in a cloned EBV DNA fragment which extends from bp 163473 across the terminal repeat to bp 3502 of the B95-8 sequence (Fig. 1, lines 4 [pRL60] and 5 [pLMP2A260S]). The truncated LMP2A encoded by this mutant would terminate at the end of the fifth transmembrane domain at an amber nonsense codon. Recombinant viruses were generated by homologous recombination following transfection of P3HR1 cells with *EcoRI*-A to rescue the EBNA2 and EBNALP deletion, pLMP2A260S to replace the wild-type LMP2 sequence, and the Z immediate-early EBV transactivator under control of a heterologous promoter to induce lytic replication (pSV-NaeZ). Culture supernatants were harvested 6 days post-transfection, filtered, and used to infect primary B lymphocytes at a multiplicity of one to five nonrecombinant viruses per cell. The infected cells were plated by limiting dilution to yield less than one LCL per microwell.

The resulting LCLs were screened for the LMP2 mutation by PCR with primers flanking the site of insertion of the nonsense codon (Fig. 1, line 3, 5'LMP2A/B and 3'LMP2A/B). Approximately 4% (4 of 103) of the LCLs were infected with virus which had the nonsense linker, as evidenced by the amplification of a 168-bp DNA fragment with primers specific for DNA on either side of the stop codon insertion site (data not shown). All four also had the 150-bp PCR product expected from wild-type LMP2 (data not shown). Previous results have shown that many or most cells infected with an EBNALP(WT) EBNA2(WT) recombinant from such an experiment are also infected with parental P3HR1 EBV (18). Uniform coinfection with P3HR1 could have occurred by chance or because the P3HR1 genomes provided wild-type LMP2A and LMP2B *in trans*, complementing the mutant LMP2A and LMP2B genes. Thus, these initial results were not definitive with regard to the central issue of the role of LMP2A and LMP2B in EBV infection.

Segregation of LMP2A and LMP2B mutant EBV recombinants. To attempt to segregate the LMP2 mutant EBV recombinant from the coinfecting P3HR1, we induced lytic-virus replication in the four coinfecting LCLs and cocultivated them with primary B lymphocytes. One of the four released virus which transformed primary B lymphocytes. Thirty-seven of the resulting progeny LCLs were subjected to PCR analysis with the primers which flanked the stop codon insertion site. Of these LCLs, 15 contained only wild-type LMP2 DNA, 4 contained mutant LMP2 DNA, and 18 contained both DNAs. The finding of 15 LCLs infected with virus having only wild-type LMP2 DNA versus 4 infected with virus having only mutant DNA initially suggested selection against mutant LMP2 recombinants because of an inability to infect or growth transform LCLs; many or most of the 15 LCLs which have wild-type LMP2 DNA are likely to be coinfecting with P3HR1. From this latter perspective, a total of 22 LCLs had mutant LMP2 and only 15 had no mutant LMP2. Rather than pursue further evaluation of this aspect of the experiments, we decided to focus on the LCLs which appeared to be infected with only mutant recombinant LMP2 genomes. If further analysis confirmed this result, and if mutant recombinant virus could be recovered from the infected LCLs, the role of LMP2 could be more directly evaluated by using the clonal mutant recombinant virus. LMP2A260S mutant recombinant virus could then be directly compared with wild-type control virus in *in vitro* lymphocyte infection and growth transformation.

LMP2A and LMP2B mutant EBV recombinants can transform primary B lymphocytes. Two LCLs infected with only

FIG. 1. Schematic representation of the LMP2A and LMP2B genes in the EBV genome. In line 1, the EBV genome circularized through the terminal repeats (TR) is depicted with the rightmost terminal *EcoRI* fragment, *D_{het}*, and the leftmost terminal fragment, I, indicated. Line 2 depicts the LMP2 transcript and the splicing which results in the LMP2A and LMP2B mRNAs. Open exons represent noncoding segments of the mRNA, and solid exons represent coding regions. Line 3 indicates the location of primers used in PCR analysis. Lines 4 and 5 depict the EBV DNA in plasmid clones used in the construction of the LMP2A mutants. Relevant restriction sites are as shown. The nonsense linker which contains an *XbaI* site was inserted into the unique *SalI* site of pRL60 by blunt-end ligation. In line 6, the *SacI*-*KpnI* fragment used as a probe in Fig. 3 is indicated.

a LMP2A260S recombinant (260SJ.7 and 260SJ.31) were induced, and the resultant filtered virus was used to infect primary B lymphocytes under conditions where there was fewer than one transforming virus per five microwells. Wild-type recombinant virus was used to infect primary B lymphocytes in parallel. Primary B lymphocytes infected with mutant recombinant virus grew out as rapidly as cells infected with wild-type virus, and mutant recombinant virus-infected LCLs were indistinguishable in their growth from wild-type recombinant virus-infected LCLs. As expected, LCLs arising following infection with LMP2A260S recombinants had only genomes with the LMP2A260S mutation (Fig. 2A, lanes 260SJ.7.A to 260SJ.7.C and 260SJ.31.A to 260SJ.31.C). As demonstrated by PCR in Fig. 2C, no wild-type LMP2A DNA could be detected in these cell lines or in the 260SJ.7 and 260SJ.31 cell lines from which the mutant recombinant virus was derived, using primers which are specific for the wild-type DNA. PCR with these primers is sufficiently sensitive to detect 1 in 1,000 wild-type LMP2 DNAs (Fig. 2B).

Confirmation of the genotype in mutant recombinant virus-infected cells. The LMP2A260S mutation in the infected LCLs was further confirmed by nucleotide sequence analysis and by Southern blot hybridization. PCR-amplified DNAs from the linker insertion site of two LMP2A260S-infected LCLs were sequenced. The sequence of the amplified DNAs was exactly that expected from the EBV and linker sequences and the methods of construction used (data not shown). To confirm that homologous recombination occurred and had replaced the wild-type LMP2A allele with

the LMP2A260S mutation in the expected context in the recombinant P3HR1 genomes, we subjected LCL DNA to Southern blot analysis (Fig. 3). If the mutant LMP2A/LMP2B had replaced the wild-type DNA, the *EcoRI* I fragment, which includes the insertion site, should have a new *XbaI* site (Fig. 1, line 5). DNA from representative mutant or wild-type recombinant- or nonrecombinant-transformed LCLs was digested with *EcoRI* or with *EcoRI* and *XbaI*. A Southern blot of the digested DNA was then probed with the labeled *SacI*-*KpnI* fragment (Fig. 1, line 6). The resulting autoradiogram is shown in Fig. 3. Wild-type EBV DNA, both P3HR1 and B95-8 strains, has the *EcoRI* I fragment. In addition, because the probe includes the terminal repeats, the variable-sized *EcoRI* *D_{het}* fragments (whose size depends on the number of terminal repeats) are also detected. Each LMP2A260S recombinant virus-infected LCL has a new *XbaI* site, which divides the *EcoRI* I fragment into 3,526- and 651-bp fragments in the *EcoRI*-*XbaI* double digests (Fig. 3, lanes 12 to 14, fragments indicated by arrows). *XbaI*-resistant *EcoRI* I fragments were detected only in the wild-type control DNAs (Fig. 3, lanes 9 to 11 and 15) even after long exposures of the blot (data not shown).

Latent and lytic-cycle EBV gene expression in LCLs infected with LMP2A260S mutant EBV recombinants. By using a polyclonal affinity-purified antibody which recognizes the LMP2A unique amino-terminal domain, wild-type LMP2A was not detected in the LMP2A260S mutants (Fig. 4, lanes 260SJ.7 and 260SJ.31) whereas a truncated LMP2A product of 44,000 Da was observed. The predicted size of the truncated product is 27,716 Da, considerably smaller than

FIG. 2. PCR analysis of the wild-type and mutant LMP2A in EBV recombinant-infected LCLs. (A) Ethidium bromide-stained PCR products from LCL DNA obtained by using primers which flank the *SacI* site at which the stop codon was inserted into LMP2A and LMP2B. Lanes P3HR1-16 and LCL1 contain the expected wild-type product of 150 bp. Lanes 260SJ.31.C, 260SJ.31.B, 260SJ.31.A, 260SJ.31, 260SJ.7.C, 260SJ.7.B, 260SJ.7.A, and 260SJ.7 have only the amplified 168-bp product as expected from LCLs singly infected with EBV carrying only LMP2A260S DNA. Lanes pLMP2A260S and pRL60 are the results of control amplifications of the wild-type (pRL60) or mutant (pLMP2A260S) plasmids. (B) To assess the sensitivity of PCR detection of wild-type genomes in a background of LMP2A260S recombinant genomes, dilutions of genomic DNA with EBV-negative BJAB DNA were added to a constant amount of genomic DNA from mutant LCL 260SJ.31.A and amplified for 40 cycles with an LMP2A and LMP2B insertion site-specific primer which has a single-nucleotide mismatch with the LMP2A260S DNA at its 5' end. (C) Amplifications of LCL DNA and plasmid controls with the LMP2A wild-type-specific primers. Sizes of the relevant *HaeII* restriction fragments of ϕ X174 are indicated in base pairs.

the 44,000 Da observed. This anomalous migration is not surprising on the basis of observed migration of a previously described cDNA LMP2A deletion mutant, LMP2Ad168-365 (16), which has an apparent molecular size of 45,000 Da but a predicted size of 31,896 Da (16). (Compare 44,000 and 45,000 Da with 27,716 and 31,896 Da for the observed and predicted sizes of LMP2A260S and LMP2Ad168-365, respectively.) LMP2A was detected in wild-type-transformed LCLs (Fig. 4, lane LCL2), including a P3HR1 EBNA1P and EBNA2 recombinant derived in parallel with these experiments (Fig. 4, lane WT111.1). As expected, LMP2A was not detected in the EBV-negative BJAB cell line (Fig. 4, lane BJAB). LMP2B expression or expression of the truncated LMP2B product in these cells could not be detected since

FIG. 3. Southern blot analysis of LMP2A and LMP2B alleles in wild-type and mutant EBV-immortalized LCLs. DNA from representative LCLs was digested with *EcoRI* or *EcoRI-XbaI* and probed with the ³²P-labeled *SacI-KpnI* fragment indicated in Fig. 1, line 6. In *EcoRI* digests, the probe detected the wild-type *EcoRI* D_{het} fragment in all the cell lines tested (lanes 2 to 15). The *EcoRI* fragment varies in size because of differences in the number of terminal repeats contained within the fragment (Fig. 1, line 1). The probe detected the wild-type *EcoRI* I fragment in all cell lines tested (lanes 2 to 8). In the double digests with *EcoRI* and *XbaI*, the probe detected the same wild-type fragment in LCL2, P3HR1-16, and WT111.6 and WT111.27 (two P3HR1 EBNA1P and EBNA2 recombinants) because of the absence of any *XbaI* sites within the wild-type fragment (lanes 9 to 11 and 15). The 3,526- and 651-bp fragments (indicated by arrows) results from cleavage at the inserted *XbaI* nonsense linker at the *Bam*HI site shown in Fig. 1, line 5. In three LCLs which, by PCR analysis, were infected with only mutant LMP2A alleles, only the 3,526- and 651-bp fragments are detected in the *EcoRI-XbaI* double digests (lanes 13 to 15). The absence of the wild-type fragment indicates that these LCLs contain only mutant alleles of LMP2A.

currently available antibodies recognize only the amino-terminal cytoplasmic domain unique to LMP2A (17). Presumably, the LMP2B product would be similarly truncated since the same DNA encodes this part of both proteins.

Aside from LMP2A, EBV gene expression in LCLs infected with and immortalized by LMP2A260S mutant EBV recombinants was identical to that in wild-type controls. A human serum sample reactive with the EBNA3 proteins demonstrated similar EBNA1 and EBNA2 expression in wild-type and mutant LMP2A EBV recombinant-infected LCLs (Fig. 5A). The EBNA3 proteins are not as readily detected in the P3HR1-based wild-type or LMP2A260S mutant recombinants as in the B95-8-transformed LCL1 (Fig. 5A, lane LCL1), because of type-specific differences between the B95-8 and P3HR1 EBNA3s. LMP1 expression was similar in both wild-type and mutant EBV-infected LCLs (Fig. 5B).

In double immunofluorescence with LMP1 and LMP2 antibodies, the truncated LMP2A gene product colocalized

FIG. 4. Wild-type LMP2A is absent and only truncated LMP2A is detected in the LMP2A mutant EBV recombinant-infected LCLs. Extracts of approximately 10^6 cells were electrophoresed, transferred to nitrocellulose, and reacted with purified LMP2A antibodies as described in Materials and Methods. LMP2A migrates just below the 66-kDa size marker. The abundant, truncated LMP2A is evident just above the 43-kDa size marker. BJAB is an EBV-negative B-lymphoma cell line; WT111.1 is a P3HR1 EBNA2-EBNA1 recombinant control; 260SJ.7 and 260SJ.31 are LMP2A and LMP2B mutants; and LCL2 is a B95-8-transformed LCL. Sizes of protein standards are indicated in kilodaltons.

with the LMP1 patch in the plasma membrane of three LMP2A260S mutant-infected LCLs. A similar colocalization of LMP1 and LMP2A in the plasma membrane was observed in three P3HR1 EBNA2 and EBNA1 wild-type control-infected LCLs.

Lytic-cycle gene expression was studied following transfection with pSVNaeZ and TPA (12-*O*-tetradecanoylphorbol-13-acetate) treatment. Seven LMP2A260S mutant-infected and five wild-type control-infected LCLs were analyzed for their response to lytic-cycle induction. Five days after transfection and addition of TPA, cells were stained by immunofluorescence with 2L10, a monoclonal antibody directed against gp350/220. A total of 11, 9.7, 8.3, 8.2, 6, 3, and 2.4% (average, 6.9%) of the LMP2A260S mutants stained brightly for gp350/220 expression, whereas 11, 11, 7.5, 7.4, and 6% (average, 8.6%) of the wild-type controls were positive for gp350/220 expression. Thus, the LMP2A and LMP2B mutation does not significantly affect EBNA or LMP gene expression or the progression from latent to lytic virus gene expression following induction of lytic virus infection.

Comparative growth of LMP2A260S mutant- and wild-type-infected LCLs. The initial transformation efficiency and growth characteristics of the LMP2A260S mutant and wild-type control recombinant viruses were investigated. EBV replication was induced in the LMP2A and LMP2B mutant or wild-type control EBV recombinant-infected cells, which had similar levels of permissivity as assayed by EBV gp350 expression. Dilutions of the resultant released virus from the induced cells were filtered and used to infect primary B lymphocytes, and the infected cells were plated into microwells in assays of transforming activity. Similar numbers of wells were positive for LCL outgrowth, and the resultant LCLs grew out similarly and were indistinguishable in their

FIG. 5. Latent gene expression in the LMP2A and LMP2B mutants. Extracts of approximately 5×10^5 cells were separated in denaturing polyacrylamide gels, transferred to nitrocellulose, and blotted with either an EBV immune human serum (A) or anti-LMP1 monoclonal antibodies (B). BJAB is an EBV-negative B-lymphoma cell line; B95-8 is a marmoset line partially permissive for viral replication; P3HR1-16 is the HH514-16 subclone of P3HR1; LCL1 is a B95-8-transformed LCL; 260SJ.6 and 260SJ.27 are the P3HR1 EBNA2-EBNA1 control recombinants P3HR1 recombinant WT111.6 and WT111.27, respectively; and 260SJ.7 through 260SJ.31.C are LMP2A and LMP2B mutant LCLs. The positions of the EBNA3, EBNA2, EBNA1, LMP1, and molecular size markers are indicated in kilodaltons.

growth characteristics as they were expanded to larger cultures. Two mutant-infected LCLs released 3×10^2 and 12×10^2 virus transforming units per percent gp350 positive cells, whereas two wild-type-infected LCLs released 6×10^2 and 11×10^2 virus transforming units per percent gp350 positive cells. PCR analysis of the resulting LCL DNA confirmed the presence of the stop codon mutation in the mutant-infected LCLs.

To further investigate the effect of the LMP2A mutation on the growth of transformed LCLs, we plated newly transformed mutant or wild-type control recombinant virus-infected LCLs at various cell concentrations and in media supplemented with 10, 1, 0.1, or 0% calf serum. Growth was then assayed 10 days later by microscopic examination and monitoring of medium pH. At a seeding density of 5×10^3 or 2.5×10^4 cells per well, both the mutant- and wild-type-infected LCLs were unable to grow or grew poorly in wells containing less than 10% serum. All LCLs grew well at 2.5×10^4 cells per well in 10% serum, while the mutant-infected LCLs tended to grow better than the wild-type-infected LCLs at 5×10^3 cells per well in 10% serum. At 1×10^5 cells per well, the mutant-infected LCLs and one wild-type-infected LCL were able to grow in all wells, even with serum concentrations as low as 0.1%. Overall, mutant recombinant-infected LCLs were at least as resistant to plating at low cell density or in media supplemented with low serum concentrations as wild-type-infected LCLs were. These data are summarized in Table 1.

Anchorage-independent growth of wild-type- and LMP2

TABLE 1. Serum and cell-dependent growth of LMP2A and LMP2B mutants^a

Cell line	Growth ^b at following cell no. and serum concn (%):											
	10 ⁵ cells				2.5 × 10 ⁴ cells				5 × 10 ³ cells			
	10%	1%	0.1%	0%	10%	1%	0.1%	0%	10%	1%	0.1%	0%
260SJ.31	+++	++	+	±	+++	+	-	-	++	-	-	-
260SJ.6	+++	+++	+++	++	+++	++	-	-	+++	-	-	-
260SJ.36	+++	+++	+++	++	+++	+	-	-	++	-	-	-
260SJ.7	+++	+	+	±	+++	-	-	-	++/+	-	-	-
WT111.11	+++	++/+	±	±	+++	±	-	-	+	-	-	-
WT111.27	+++	+++	++	+	+++	-	-	-	++	-	-	-
WT111.K2	+++	+	-	-	+++	-	-	-	±	-	-	-

^a Cells were plated in 96-well tissue culture plates at either 10⁵, 2.5 × 10⁴, or 5 × 10³ cells per well in 10, 1, 0.1, or 0% serum. The wells were scored 10 days after plating.

^b Growth was scored according to the following guidelines: +++, large clumps, acidified media, clear growth; ++, medium clumps, possible acidification of media, probable growth; +, small clumps, neutral or basic media, little growth; -, no clumps, neutral or basic media, no growth.

mutant-infected LCLs was assayed by plating in soft agar over a fibroblast feeder layer. Four LMP2A260S mutants and six wild-type control-infected LCLs which had been derived and maintained in parallel within the previous 2 months were plated at 10³ cells per ml in 0.3% agarose in 4-cm wells over a fibroblast feeder layer. After 4 weeks, the wells were scored for macroscopic cell growth. The mutant-infected LCLs had a cloning efficiency of 0.5 to 1.3% (average, of 1.0%), whereas the wild-type-infected LCLs had a cloning efficiency of 0.4 to 4.1% (average, 1.7%). Thus, the cloning efficiencies are similar. These data are summarized in Table 2.

DISCUSSION

The experiments described above demonstrate that the last seven transmembrane and the cytoplasmic carboxy-terminal domains of LMP2A and LMP2B are not essential or critical for the initiation or maintenance of latent or lytic lymphocyte infection or growth transformation in vitro. Further, mutation does not significantly affect the ability of mutant recombinant-infected cells to initially grow out as LCLs, to grow at low cell density and at reduced serum concentrations, or to form colonies in soft agar. These conclusions are based on the lack of effect of a specific LMP2A and LMP2B mutation replacing the normal allele in a recombinant EBV genome. The mutation truncates the 497-amino-acid LMP2A protein at amino acid 260 and the 378-amino-acid LMP2B protein at amino acid 141 before the last seven transmembrane domains and the cytoplasmic carboxy-terminal domain. The next ATG in a favorable

context for potential reinitiation of translation is in the eight-transmembrane domain and, if used, would result in a protein which would be unlikely to associate with the rest of LMP2A and LMP2B. Infection with the mutant recombinant virus was confirmed by immunoblot, PCR, Southern blot, and DNA sequence analyses.

The amino-terminal cytoplasmic domain unique to LMP2A has similarly been demonstrated to be dispensable for initiation or maintenance of latent or lytic lymphocyte infection or growth transformation; this was done by constructing a mutant EBV recombinant with a stop codon after codon 19 (18). The only part of LMP2 which has not been tested for its importance in lymphocyte growth transformation is the part containing the first five hydrophobic transmembrane domains common to LMP2A and LMP2B. This part of LMP2 is presumably expressed in cells infected with the mutant recombinants described in this and the previous paper. Expression of this part of LMP2 could not be directly evaluated because available antisera do not react with these hydrophobic transmembrane domains. A mutant which cannot express this part of LMP2 should be formally evaluated. However, the first five transmembrane domains and the four short interspersed hydrophilic sequences have few remarkable characteristics and are unlikely to constitute a domain with significant transforming properties. Unless these first five transmembrane domains have some unexpected function when expressed alone, LMP2A and LMP2B are not necessary for any stage of B-lymphocyte infection in vitro.

The apparent lack of a phenotypic difference between mutant and wild-type LMP2 EBV recombinant-infected LCLs is surprising since other observations indicate that LMP2 is likely to be important in EBV infection. LMP2A and LMP2B are expressed in all latently infected B lymphocytes in vitro (17), infected B lymphocytes in vivo (27), and in tumor tissues from individuals with nasopharyngeal carcinoma (3, 6). Patients with nasopharyngeal carcinoma have antibodies to LMP2 (8). Further, LMP2A interacts with membrane-associated B-lymphocyte *src* family tyrosine kinases, particularly *fyn* and *lyn*. LMP2 has T-cell-reactive epitopes which can mediate cytotoxic T-cell responses and which should select against EBV genomes that express LMP2 in humans with T cells of this specificity (25).

Thus, unless the domain which includes the first five transmembrane components of LMP2 proves to be essential and sufficient for lymphocyte infection in vitro, the role of LMP2 role in EBV infection in vivo will not be obvious from

TABLE 2. Growth in soft agar of LMP2A and LMP2B mutants compared with wild-type controls

Cell line	% Cloning efficiency ^a
260SJ.27	1.3
260SJ.36	0.8
260SJ.7	1.3
260SJ.31	0.5
WT111.K10	4.1
WT111.6	0.4
WT111.K2	0.7

^a Scoring the number of cell colonies which are macroscopic after 4 weeks of growth over a fibroblast feeder layer in wells seeded with 10³ cells.

studies of lymphocyte infection in vitro, and will have to be studied in an in vivo experimental model. The putative in vivo function is likely to be related to known LMP2 properties. LMP2A might be important in modulating the effects of LMP1 or of B-lymphocyte or epithelial-cell transmembrane signaling. LMP2A associates with LMP1 in EBV-infected LCLs and in B-lymphoma cells in which both proteins are expressed under the control of heterologous promoters (16). LMP2A is also serine, threonine, and tyrosine phosphorylated and associates with *fyn* and *lyn* and other B-lymphocyte *src* family tyrosine kinases in EBV-infected LCLs (4, 16). In addition, the LMP2A mRNA is the only EBV mRNA detected in lymphocytes purified from the peripheral blood of latently infected humans (27). LMP2 mRNAs are also expressed in tumor biopsy specimens from patients with nasopharyngeal carcinoma (3, 6). Thus, LMP2 might be important for persistence or reactivation in lymphoid or epithelial tissues in humans. Transfection with the Z transactivator and treatment with TPA bypass normal stimuli for reactivation which might be present in vivo. Such hypotheses will require testing in experimental models of human EBV infection such as SCID mice, cottontop tamarins, and Old World primates.

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