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Epstein–Barr virus latent membrane protein 1 is essential for B-lymphocyte growth transformation

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ABSTRACT **The gene encoding latent-infection membrane protein 1 (LMP1) was specifically mutated in Epstein–Barr virus (EBV) recombinants by inserting a nonsense linker after codon 9 or codon 84 or into an intron 186 bp 3′ to the latter insertion site. EBV recombinants with the LMP1 intron mutation were wild type for LMP1 expression and for growth transformation of primary B lymphocytes. In contrast, EBV recombinants with the mutations in the LMP1 open reading frame expressed N-terminally truncated crossreactive proteins and could initiate or maintain primary B-lymphocyte transformation only when wild-type LMP1 was provided in trans by a coinfecting, transformation-defective EBV, P3HR-1. These data indicate that LMP1 is essential for EBV-mediated transformation of primary B lymphocytes, that the first 43 amino acids are critical for LMP1's function, and that codon 44-initiated LMP1 does not have a dominant negative effect on transformation.**

Epstein–Barr virus (EBV) causes a lymphoproliferative disease in immune-deficient humans and is associated with Burkitt lymphoma (BL), Hodgkin disease, and nasopharyngeal carcinoma. EBV efficiently establishes a nonreplicative infection in primary primate B lymphocytes and expresses six nuclear proteins [EBV-encoded nuclear antigens (EBNAs)], three integral membrane proteins [latent membrane protein 1 (LMP1), LMP2A, and LMP2B], and two small RNAs (EBERs). EBV-infected cells proliferate indefinitely thereafter and are tumorigenic in SCID mice and in marmosets (for review, see refs. 1 and 2). Four EBNAs have been shown to be critical for B-lymphocyte growth transformation (3–6).

To evaluate whether LMP1 is essential for primary B-lymphocyte growth transformation, this study compares the transforming ability of EBV recombinants which have a nonsense linker inserted early in the LMP1 open reading frame (ORF) with that of recombinants which have the same linker inserted into an adjacent LMP1 intron and express wild-type (WT) LMP1. Previously, single-gene transfer experiments indicated that LMP1 is an important effector of changes in cell growth. When expressed in immortalized rodent fibroblasts, LMP1 induces altered morphology, reduced serum dependence, loss of contact inhibition, anchorage-independent growth, and tumorigenicity in nude mice (7–9). In EBV-negative BL cells, LMP1 induces many of the phenotypic changes seen in EBV-infected primary B lymphocytes: increased villous projections, growth in tight clumps, and increased expression of activation and adhesion molecules and of the *bcl-2* protooncogene (10, 11). LMP1 also affects the differentiation, morphology, and growth of human and rodent epithelial cells (12–14).

MATERIALS AND METHODS

Cell Lines and Virus. The HH514-16 subclone of the P3HR-1 BL cell line and the B95-8 cell line were gifts from George Miller of Yale University. LMPΔS1 and LMPΔS23 cell lines stably express codon 44-initiated LMP1 (15).

Construction of LMP1 Mutants, Generation of EBV Recombinants, and Passage of EBV Recombinants. The LMP1 gene in the *Eco*RI D_{het} fragment (nt 159,853–172,282 in the EBV genome; ref. 17) was mutated by inserting a nonsense oligonucleotide (5′-CTAGTCTAGACTAG-3′) into an *Sfi* I site (nt 169,441; after codon 9), *Xca* I site (nt 169,219; after codon 84), or *Bgl* II site (nt 169,033; in an intron). EBV recombinants were generated by electroporating 10^7 P3HR-1 cells with 20 μg of LMP1MS9, LMP1MS84, or LMP1WT(S) DNA, 10 μg of WT EBNA LP/2 DNA (*Eco*RI A, nt 7315–69,119), and 25 μg of pSVNaeIZ (an expression vector for the EBV lytic-infection transactivator BZLF1) (16, 18). T-cell-depleted mononuclear cells from healthy donors were infected with virus, seeded into 96-well plates at 5×10^4 per well, and maintained in RPMI 1640 with 10% fetal bovine serum (16, 18). Lytic infection was induced in these lymphoblastoid cell lines (LCLs) by electroporation with pSVNaeIZ and addition of phorbol ester to the medium (19). T-cell-depleted mononuclear cells were infected with filtered virus or by cocultivation with 2.5×10^4 lethally irradiated (8000 rads; 1 rad = 0.01 Gy) LCL cells per well (16). The absence of an endogenous WT EBV in the primary lymphocytes was demonstrated by plating uninfected primary B lymphocytes in parallel cultures. No spontaneous LCL outgrowth was detected.

Analysis of LCLs. LMP1 was detected with the S12 (anti-LMP1) monoclonal antibody (10, 20, 21).

RESULTS

Construction of EBV Recombinants with a Mutated LMP1 Gene. A nonsense linker was inserted after LMP1 codon 9 (LMP1MS9) or 84 (LMP1MS84) or into an LMP1 intron [LMP1WT(S)] in the *Eco*RI D_{het} genomic fragment of EBV (Figs. 1 and 2). LMP1 consists of a 24-aa cytoplasmic N terminus, six hydrophobic transmembrane domains separated by short reverse turns, and a 192-aa cytoplasmic C terminus (Fig. 1 and ref. 22).

EBV recombinants were generated by cotransfecting a mutated *Eco*RI D_{het} DNA fragment into P3HR-1 cells along with another EBV DNA fragment to allow marker rescue of the transformation-incompetent P3HR-1 EBV (16). P3HR-1 cells are latently infected with a lytic-replication-competent EBV which lacks a DNA segment which includes part of EBNA LP and all of EBNA 2 DNA and is consequently transformation-defective (3–5). Transfection of P3HR-1 cells with a cloned WT EBV DNA fragment which encodes EBNA LP/2 and spans the P3HR-1 deletion site, and simultaneous induction of lytic EBV replication, results in 10^7–10^8 nonre-

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Abbreviations: EBV, Epstein–Barr virus; LMP, latent membrane protein; EBNA, EBV-encoded nuclear antigen; LCL, lymphoblastoid cell line; BL, Burkitt lymphoma; WT, wild type; ORF, open reading frame; CRP, crossreactive protein.

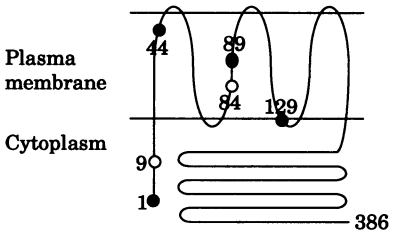

FIG. 1. Structure of LMP1 in the plasma membrane (22, 23). LMP1MS9 DNA is predicted to stop translation after amino acid 9 (○) and is believed to reinitiate translation at methionine 44, 89, or 129 (●). LMP1MS84 DNA is predicted to stop translation after amino acid 84 and is believed to reinitiate translation at methionine 89 or 129.

combinant P3HR-1 EBV virions and 1–2 × 10^2 WT EBNA LP/2 EBV recombinants (4, 16). When this virus preparation is used to infect 5 × 10^7 primary B lymphocytes and the infected cells are plated into 10^3 wells, WT EBNA LP/2 EBV recombinants can be specifically and clonally selected by their ability to transform a primary B lymphocyte into an LCL in 10–20% of the wells. Lymphocytes which are not infected with a WT EBNA LP/2 recombinant die within 14 days in culture. Based on previous results with a larger EBV DNA fragment, up to 10% of the WT EBNA LP/2 EBV recombinants would be expected to incorporate the cotransfected, mutated LMP1 gene by second-site homologous recombination (6, 16, 18). If LMP1 is not essential for growth transformation, these recombinants will transform primary B lymphocytes into LCLs. If LMP1 is essential for growth transformation, WT EBNA LP/2 EBV recombinants with an LMP1MS9 or -84 mutation might still be recovered in LCLs because at least half of the primary B lymphocytes are initially coinfected with nonrecombinant P3HR-1 EBV, which would provide WT LMP1 in trans (Fig. 2; refs. 6, 16, and 18). Coinfection with nonrecombinant P3HR-1 occurs because 10^7 to 10^8 P3HR-1 EBV virions result from each transfection and the entire virus preparation is used to infect 5 × 10^7 lymphocytes.

Recovery of EBV Recombinants with Specifically Mutated LMP1 DNA. LCLs infected with EBV recombinants with mutated LMP1 DNA were first identified through detection of the inserted linker DNA by PCR (Fig. 3 *A–C*). The fraction of LCLs infected with EBV recombinants with mutated LMP1 DNA was 8/154 (5%) from four cotransfections of P3HR-1 cells with LMP1MS9, 3/156 (2%) from two cotransfections with LMP1MS84, and 5/156 (3%) from two cotransfections with LMP1WT(S). Southern blots of DNA from these LCLs were consistent with incorporation of mutated LMP1 DNA into the EBV genome by homologous recombination (Fig. 3*D*). An LMP1 DNA probe hybridized to a fragment of expected size (2.4 kb) after cleavage with *Mlu* I. Double digestion with *Mlu* I and *Xba* I resulted in two smaller fragments of expected size due to the inserted oligonucleotide (Fig. 3*D*). Similar results were obtained for each of the DNAs with *Bam*HI and with *Bam*HI and *Xba* I and in some instances with *Eco*RI (data not shown) (*Bam*HI and *Eco*RI cut EBV DNA 6.7 kb and 2.7 kb upstream and 0.5 kb and 7.3 kb downstream of the *Mlu* I sites).

All 11 LCLs infected with an LMP1MS9 or LMP1MS84 EBV recombinant also had WT LMP1 DNA which lacked the *Xba* I linker by PCR and Southern blot (Fig. 3 and data not

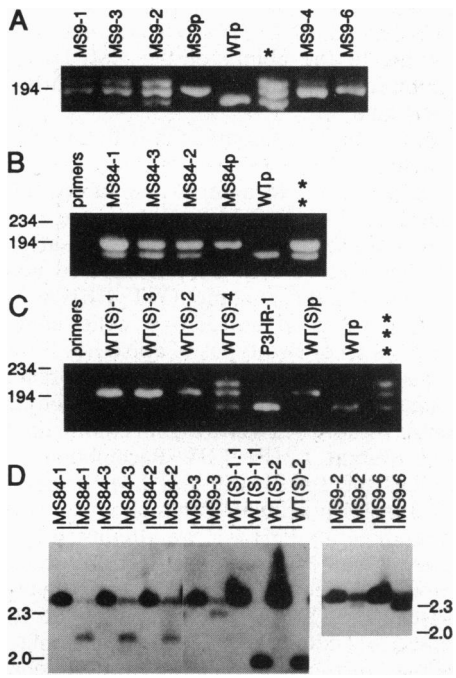

FIG. 3. PCR and Southern blot analyses of LMP1 DNA in LCLs infected with an EBV recombinant. (*A–C*) Ethidium bromide-stained agarose gels of PCR DNA products. (*A*) LMP1MS9 primers (nt 169,327–169,349 and nt 169,508–169,486; ref. 17) amplify a 182-bp DNA from WT LMP1 DNA and a 193-bp DNA from LMP1MS9 DNA. (*B*) LMP1MS84 primers (nt 169,072–169,091 and nt 169,253–169,234) amplify a 182-bp DNA from WT LMP1 DNA and a 196-bp DNA from LMP1MS84 DNA. (*C*) LMP1WT(S) primers (nt 168,919–168,938 and nt 169,100–169,081) amplify a 182-bp DNA from WT LMP1 DNA and a 200-bp DNA from LMP1WT(S) DNA. PCR mixtures with primers but no cell DNA were loaded in lanes marked "primers." LMP1MS9, LMP1MS84, LMP1WT(S), and WT LMP1 plasmid DNA clones were PCR-amplified and loaded in lanes marked MS9p, MS84p, WT(S)p, and WTp, respectively. PCR DNA products from cloned LMP1MS9, LMP1MS84, or LMP1WT(S) plasmid DNA were annealed with PCR DNA products from cloned WT LMP1 plasmid DNA and loaded in lane *, **, or ***. The third, more slowly migrating fragment in the * and *** lanes is a heteroduplex of WT and mutated LMP1 DNA PCR products (16, 19). LCLs MS9-4 and MS9-6 (in *A*) have more heteroduplex than WT DNA PCR product. DNA size markers (bp) are indicated at left. (*D*) Southern blot analysis of LCLs for LMP1 DNA. DNA was size-separated and probed with a radiolabeled 2.4-kb *Mlu* I fragment from WT LMP1 DNA. DNA samples were loaded in pairs as *Mlu* I-digested (left) and *Xba* I/*Mlu* I double-digested (right). Due to the inserted oligonucleotide, *Xba* I cleaves the 2.4-kb *Mlu* I fragments containing LMP1MS9 (2.3 kb and 0.1 kb), LMP1MS84 (2.1 kb and 0.3 kb), and LMP1WT(S) (1.9 kb and 0.5 kb). Fragments ≤ 0.5 kb are not shown. DNA size markers (kb) are indicated at left and right.

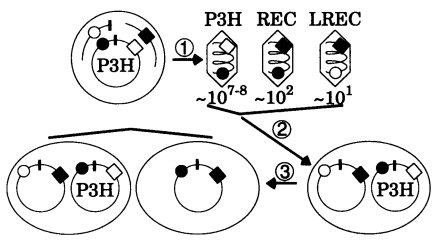

FIG. 2. Generation and analysis of LMP1MS9 or LMP1MS84 recombinant EBV. ●, Wild-type LMP1 DNA; ○, mutated LMP1 DNA; ■, WT EBNA LP/2 DNA; □, P3HR-1 deletion; ‖, EBV terminal repeat; P3H, P3HR-1 EBV; REC, WT EBNA LP/2 EBV recombinant; LREC, WT EBNA LP/2–LMP1MS9 or -84 EBV recombinant. Step 1: P3HR-1 cells were cotransfected with mutated LMP1 DNA and WT EBNA LP/2 DNA and lytic infection was induced. Step 2: Primary B lymphocytes were infected with virus and the resultant LCLs were analyzed for mutated LMP1 DNA. Step 3: LCLs coinfected with an LMP1MS9 or LMP1MS84 recombinant and P3HR-1 were induced for lytic infection, and primary B lymphocytes were infected with progeny virus. Progeny LCLs that arose were analyzed for mutated LMP1 DNA. Estimated numbers of each type of virus are indicated (6, 16, 18).

shown). The finding of the appropriate size WT and mutated EBV DNA fragments after *Mlu* I, *Bam*HI, or *Eco*RI digestion indicated that the mutated and WT LMP1 genes were each in the appropriate context on separate EBV genomes. The presence of the parental P3HR-1 genome in these cells was confirmed by PCR analysis (data not shown) with primers that flank both sides of the 6.6-kb P3HR-1 EBNA LP/2 deletion and amplify a 256-bp DNA fragment from P3HR-1 (6). These LCLs are therefore coinfected with a WT EBNA LP/2 and LMP1MS9 or -84 EBV recombinant and with a nonrecombinant P3HR-1 EBV which has WT LMP1 DNA (and is deleted for WT EBNA LP/2 DNA; Fig. 2).

By similar analyses (Fig. 3 and data not shown), one of the five LCLs infected with an LMP1WT(S) EBV recombinant had no coinfecting P3HR-1 EBV. Over the first 8 months in culture, three of the other four LCLs which were initially coinfected with an intron control [LMP1WT(S)] recombinant and P3HR-1 (WT LMP1) lost WT LMP1 DNA through the loss of coinfecting P3HR-1. This indicates that P3HR-1 was not contributing to the ability of the LMP1WT(S) intron control recombinant to maintain primary B-lymphocyte growth transformation.

In contrast, seven LCLs coinfected with an LMP1MS9 or -84 recombinant and P3HR-1 which could be passaged in continuous culture for >18 months maintained the P3HR-1 genome. One LCL lost the LMP1MS9 gene as the result of secondary recombination between the two different viral genomes in the infected cell. [A cell infected with such a secondary recombinant genome (WT EBNA LP/2–WT LMP1) should have an advantage over cells requiring post-mitotic transmission of two types of episomes for continued growth.] Thus, LMP1MS9 and LMP1MS84 recombinants probably cannot maintain primary B-lymphocyte growth transformation without WT LMP1 from coinfecting P3HR-1.

Passage of Mutant LMP1 EBV Recombinants. To test further whether the LMP1MS9 and LMP1MS84 recombinants could transform primary B lymphocytes without WT LMP1 from coinfecting P3HR-1, we attempted to segregate the coinfecting genomes from these LCLs by inducing lytic virus infection and infecting primary B lymphocytes with the resultant virus (Fig. 2). A low frequency of lytic virus infection could be induced in five LCLs coinfected with P3HR-1 and any of four different LMP1MS9 recombinants, in five LCLs coinfected with P3HR-1 and any of three different LMP1MS84 recombinants, and in one LCL coinfected with P3HR-1 and LMP1WT(S). Primary B lymphocytes were infected either by cocultivation with lethally irradiated LCLs or by infection with 0.45-μm-filtered supernatant virus (Fig. 2). Progeny LCLs were analyzed for mutated or WT LMP1 DNA by PCR (Table 1). No progeny LCL was infected with an LMP1MS9 or LMP1MS84 recombinant alone. Eighty-five progeny LCLs infected with an LMP1MS9 recombinant and 186 LCLs infected with an LMP1MS84 recombinant all had WT LMP1 DNA from coinfecting P3HR-1. Twenty-four of these LCLs were serially passaged for 8 months. All have maintained mutated and WT LMP1 DNA. Many (172) other progeny LCLs were infected with a WT EBNA LP/2–WT LMP1 secondary recombinant (Table 1). These WT EBNA LP/2–WT LMP1 secondary recombinants arose during lytic replication in the parental coinfected LCL. The failure to recover LCLs infected with an LMP1MS9 or LMP1MS84 recombinant alone is especially significant when one considers that LCLs coinfected with an EBV recombinant and P3HR-1 produce these viruses in excess over secondary recombinants during lytic virus infection (16). These results underscore the stringent requirement for WT LMP1 in transformation.

In contrast to these results, LMP1WT(S) intron control recombinant virus readily segregated from P3HR-1 even when virus was passaged by cocultivation of irradiated LCLs with primary B lymphocytes. The progeny LCLs were infected with either an LMP1WT(S) recombinant alone (10/15 LCLs) or a WT EBNA LP/2–WT LMP1 secondary recombinant alone (5 of 15 LCLs) (Table 1). No LCL was coinfected with an LMP1WT(S) recombinant and P3HR-1. The high frequency of segregating the WT EBNA LP/2–LMP1WT(S) recombinant (10/15) is similar to previous results with other LCLs coinfected with P3HR-1 and transformation-competent EBV recombinants (16). To establish the relevance of the previous results to the current experiments, virus replication was induced in LCLs coinfected with P3HR-1 and either of two other previously derived transformation-competent EBV recombinants (T1E3-100 or T1E3-107) which can be distinguished from P3HR-1 by PCR analysis of their EBNA 3A genes (16). Fifty-seven of 141 progeny LCLs from cocultivations with primary B lymphocytes were singly infected with a recombinant and lacked coinfecting P3HR-1. The remaining progeny LCLs were either coinfected with the recombinant and P3HR-1 (53/141) or were infected with a secondary recombinant EBV that had incorporated P3HR-1's EBNA 3A gene (31/141).

Table 1. PCR analysis for LMP1 DNA in progeny LCLs

Virus source	No. of progeny LCLs		
	Mutant alone	Mutant and WT LMP1	WT LMP1 alone
MS9-1*	0	25	38
MS9-2	0	12	6
MS9-3*	0	0	2
MS9-4	0	21	17
MS9-2.2†	0	27	7
Total	0	85	70
MS84-1*	0	14	48
MS84-2	0	12	7
MS84-3	0	8	30
MS84-2.2‡§	0	86	6
MS84-2.3‡§	0	66	11
Total	0	186	102
WT(S)-3	10	0	5

*B lymphocytes were infected only by cocultivation.
†LCL is infected with virus from MS9-2.
‡LCLs are infected with virus from MS84-2.
§Only 0.45-μm-filtered virus was used to infect B lymphocytes.

To further confirm that transformation by WT EBNA LP/2 and LMP1MS9 or -84 EBV recombinants is dependent on WT LMP1 provided by P3HR-1 in trans, primary B lymphocytes were infected with 0.22-μm-filtered virus (to minimize infection with aggregated virus) from LCL MS9-2.2, MS9-4, MS84-2.2, or MS84-2.3 and increasing amounts of exogenously added P3HR-1 virus. In each experiment, added P3HR-1 resulted in more LCLs than were obtained without added P3HR-1. Increasing amounts of exogenous P3HR-1 resulted in increased numbers of LCLs which PCR analysis showed were coinfected with LMP1MS9 or -84 recombinants and P3HR-1 EBV.

WT and Mutated LMP1 Expression in LMP1MS9 and LMP1MS84 Recombinant-Coinfected LCLs and Effects on Cell Growth. WT LMP1 was expressed in all LCLs (Fig. 4). Only WT LMP1 was detected in LCLs infected with an LMP1WT(S) recombinant, in an LCL infected with a secondary recombinant that lost the LMP1MS9 gene (Fig. 4*A*, MS9-7WT), and in LCLs infected with a WT EBNA LP/2 EBV recombinant that had WT LMP1 DNA (Fig. 4*B*, WT1; Fig. 4*D*, WT2). In comparison, LCLs coinfected with P3HR-1 and an LMP1MS9 or LMP1MS84 recombinant had WT LMP1 and CRPs. The CRPs evident in LCLs infected with an LMP1MS9 recombinant result from reinitiation of translation at methionine codons 44 and 129 (Fig. 1), which

are in a favorable context for reinitiation (24). The largest CRP (●●), present only in LMP1MS9 recombinant-infected LCLs, is identical in size to the codon 44-initiated LMP1 (Fig. 4C; LMPΔS in ref. 15). The smallest major CRP (●●●●) is identical in size to D1LMP1, which initiates translation at LMP1 methionine codon 129 (15, 25). In single-gene-transfer experiments, D1LMP1 has no transforming properties (8, 10). LCLs coinfected with an LMP1MS84 recombinant also expressed a CRP identical in size to D1LMP1. Although D1LMP1 is expressed in lytic EBV replication (25) (Fig. 4 A, B, and D, B95-8 cells), D1LMP1 expression in LCLs infected with an LMP1MS9 or LMP1MS84 recombinant is due to reinitiation, since other more abundant lytic-infection viral proteins were barely detectable in these LCLs (data not shown). A less prominent CRP (●●●) in LCLs infected with an LMP1MS9 recombinant is probably due to inefficient reinitiation of translation due to a cytosine at the −3 position of methionine codon 89 (Fig. 1) (24). This CRP is unlikely to be a degradation product, since a similar CRP was evident in an LCL infected with an LMP1MS84 recombinant (Fig. 4B, MS84-2) and not in LCLs infected with WT LMP1 recombinants. Progeny LCLs coinfected with P3HR-1 and an LMP1MS9 or LMP1MS84 recombinant expressed LMP1 and CRPs similar to the parental LCLs (Fig. 4D). P3HR-1 cells express little LMP1 since EBNA 2, a transactivator of LMP1 transcription, is deleted (26).

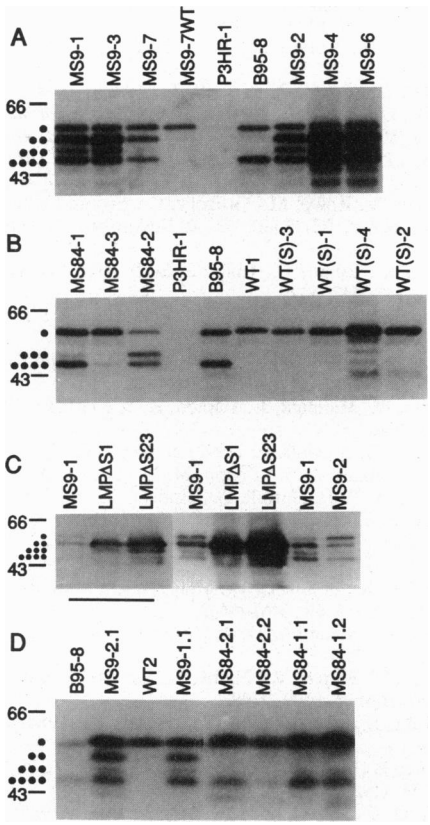

FIG. 4. Immunoblot analysis of LCLs for LMP1 (●) and cross-reactive proteins (CRPs) (●●, ●●●, and ●●●●). (A) Immunoblot analysis of LCLs coinfected with an LMP1MS9 recombinant and P3HR-1. LCL MS9-7WT is infected with a WT EBNA LP/2–WT LMP1 secondary recombinant that arose from LCL MS9-7. (B) Immunoblot analysis of LCLs coinfected with an LMP1MS84 recombinant and P3HR-1 or of LCLs infected with an LMP1WT(S) recombinant. LCL WT1 is infected with a WT EBNA LP/2–WT LMP1 recombinant. (C) Immunoblot analysis of LCLs coinfected with an LMP1MS9 recombinant and P3HR-1 or of BL cell lines expressing methionine codon 44-initiated LMP1 (LMPΔS1 and LMPΔS23). Underlined lanes are the same as the adjacent lanes with a shorter exposure time. (D) Immunoblot analysis of second generation LCLs coinfected with an LMP1MS9 or LMP1MS84 recombinant and P3HR-1. LCL WT2 is infected with a WT EBNA LP/2–WT LMP1 recombinant. B95-8 cells express WT LMP1 (●) and D1LMP1 (●●●●). Size markers (kDa) are indicated at left. Electrophoresis was done with SDS/8% polyacrylamide gels (A, B, and D) or SDS/5–18% polyacrylamide gels (C).

Immunofluorescent staining of fixed LCLs infected with an LMP1WT(S) recombinant and LCLs coinfected with P3HR-1 and an LMP1MS9 or LMP1MS84 recombinant revealed that LMP1 was tightly patched and capped in the plasma membrane, similar to LCLs infected with a WT EBNA LP/2–WT LMP1 EBV recombinant (Fig. 5D and ref. 23). LCLs coinfected with an LMP1MS9 recombinant and P3HR-1 also had diffuse staining of the plasma membrane and cytoplasm (Fig. 5A). LCLs coinfected with an LMP1MS84 recombinant and P3HR-1 also had diffuse cytoplasmic staining but had less or no diffuse plasma membrane staining (Fig. 5C). Diffuse plasma membrane staining was previously noted in BL cells that express codon 44-initiated LMP1 (Fig. 5B), and diffuse cytoplasmic staining was previously noted in BL cells that express codon 129-initiated LMP1 (10, 15). Thus, the staining observed in these infected LCLs is that expected from cells expressing LMP1 and codon 44- or 129-initiated LMP1.

Despite abundant CRP expression, the time to initial outgrowth of the LCLs coinfected with an LMP1MS9 or -84 recombinant and P3HR-1 did not differ from that of LCLs infected with an LMP1WT(S) recombinant. The subsequent doubling time in culture and the cell viability were also similar. This indicates that the CRPs do not exert an obvious negative effect on cell growth.

DISCUSSION

These experiments provide direct evidence that LMP1 is essential for EBV-induced growth transformation of primary B lymphocytes. The evidence is compelling. Specifically mutated EBV recombinants with nonsense linker insertions in the LMP1 ORF could not transform primary B lymphocytes, while control recombinants, with the linker in an adjacent intron, transformed primary B lymphocytes with the efficiency expected for a WT EBV recombinant. WT EBNA LP/2 and LMP1WT(S) intron control recombinants did not require complementation with the transformation-defective

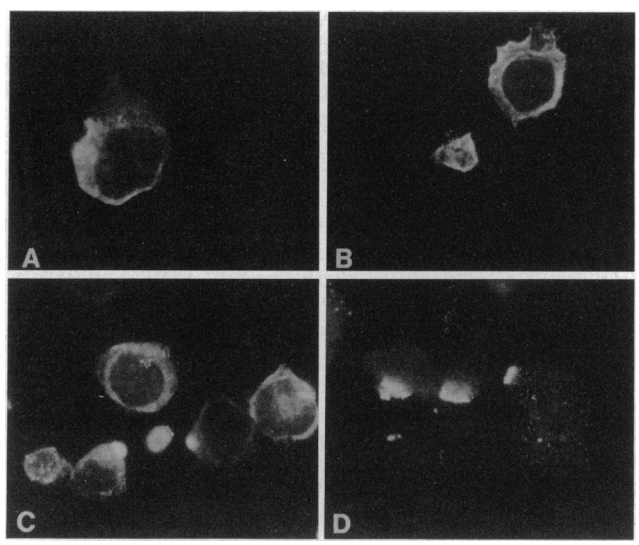

FIG. 5. Immunofluorescent staining with anti-LMP1. (A) LCL coinfected with LMP1MS9 recombinant and P3HR-1. (B) BL cell line expressing methionine 44-initiated LMP1 [LMPΔS (15)]. (C) LCL coinfected with LMP1MS84 recombinant and P3HR-1. (D) LCL infected with EBV recombinant expressing only WT LMP1. (×430.)

P3HR-1 virus to maintain or initiate B-lymphocyte growth transformation. In contrast, all WT EBNA LP/2 and LMP1MS9 or -84 recombinants required P3HR-1 to provide WT LMP1 in order to initiate and maintain growth transformation. Seven of these mutated LMP1 ORF recombinants could be passaged sequentially to primary B lymphocytes. In >300 progeny LCLs infected with the LMP1MS9 or -84 recombinants, transformation was dependent on coinfection of the B lymphocytes with P3HR-1. Further, the transfected EBV DNA used to construct the LMP1 mutant recombinants inserted into the correct site in the EBV genome and replaced the WT LMP1 gene. The only other EBV gene expressed in latent infection which could have been altered in the process of prokaryotic cloning or EBV recombination is the LMP2 gene, whose promoters and first exons are included in the cloned EBV DNA. The LMP2 gene is not essential for primary B-lymphocyte infection, for growth transformation, or for lytic virus replication (27). Moreover, the possibility that the LMP1MS9 or -84 recombinants differ from the LMP1WT(S) recombinants (which were derived in parallel) at some putative other site in their genomes is highly unlikely. With induction of lytic replication in the coinfected LCLs, recombination between the two genomes frequently occurred, resulting in WT EBNA LP/2–WT LMP1 secondary recombinants. If there were a putative (albeit unlikely) mutation at some other site in the WT EBNA LP/2–LMP1MS9 or -84 recombinant genomes that was responsible for the inability of these recombinants to transform cells, some secondary recombinants should have resulted which carried the LMP1MS9 or -84 genes and the WT allele for the putative mutated gene from P3HR-1. From seven independent WT EBNA LP/2 and LMP1MS9 or -84 recombinant virus-coinfected LCLs, 172 secondary recombinant infected LCLs were derived which transformed primary B lymphocytes, and all had WT LMP1 DNA.

The finding that LMP1MS9 recombinants express high levels of codon 44-initiated LMP1 and still require WT LMP1 from coinfecting P3HR-1 is evidence that codon 44-initiated LMP1 is not sufficient for growth transformation of primary B lymphocytes. This is also evidence that the N terminus and first transmembrane domain of LMP1 are critical to LMP1-mediated primary B-lymphocyte growth transformation (Fig. 1). Further, these data are consistent with the previous findings that codon 44-initiated LMP1 did not confer anchorage-independent growth on rodent fibroblasts and did not induce many of the activation markers induced by WT LMP1 in BL cells (15, 28). The inability of codon 44- or 129-initiated LMP1s to transform primary B lymphocytes and their altered distribution is consistent with the hypothesis that WT LMP1 acts in a patch or cap in the plasma membrane to provide a constitutive activating signal in B lymphocytes. It is also important to note that these CRPs do not have a dominant negative effect on transformed cell growth.

Studies using EBV recombinants to investigate the roles of EBV genes expressed in transformed B lymphocytes have shown that these genes fall into two categories. Inactivating mutations of the EBNA 3B, LMP2A, LMP2B, or EBER genes have no discernable effect on the ability of EBV recombinants to transform primary B lymphocytes (18, 19, 27, 29). In contrast, mutations of the EBNA 2, EBNA LP, EBNA 3A, and EBNA 3C genes, and now of the LMP1 gene, significantly affect the ability of EBV recombinants to transform primary B cells (3–6). Thus, these genes are critical or essential for primary B-cell transformation *in vitro*.

Future molecular genetic analysis with EBV recombinants should more precisely define the key components of the N-terminal 43-aa domain and of other potentially critical domains of LMP1. This will facilitate delineation of biochemical interactions important in LMP1-activation pathways. Since LMP1 is expressed in EBV-associated lymphoproliferative disease, Hodgkin disease, and nasopharyngeal carcinoma (30–33), such work may lead to insights into therapy for EBV-associated malignancies.

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