Phenotypes of Epstein-Barr Virus LMP1 Deletion Mutants Indicate Transmembrane and Amino-Terminal Cytoplasmic Domains Necessary for Effects in B-Lymphoma Cells

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The Epstein-Barr virus (EBV) latent infection membrane protein 1 (LMP1) has previously been shown to cause EBV-negative B-lymphoma cells to grow in large clumps and to alter expression of surface activation and adhesion molecules (D. Wang, D. Liebowitz, F. Wang, C. Gregory, A. Rickinson, R. Larson, T. Springer, and E. Kieff, *J. Virol.* 62:4173-4184, 1988; F. Wang, C. Gregory, C. Sample, M. Rowe, D. Liebowitz, R. Murray, A. Rickinson, and E. Kieff, *J. Virol.* 64:2309-2318, 1990). In order to identify functional elements in the amino-terminal cytoplasmic domain and the first four transmembrane domains which were previously shown to be essential for LMP1 activity, three smaller deletion mutants were constructed and tested for their activity in B-lymphoma cells. The results of the present study indicate that the amino-terminal cytoplasmic domain, the first transmembrane domain, and the third and fourth transmembrane domains each contribute to LMP1's effects on B lymphocytes.

Epstein-Barr virus (EBV) is associated with Burkitt's lymphoma and lymphoproliferative disorders in immunocompromised patients. In vitro EBV infection of normal B lymphocytes results in the activation of cells and in continuous cell proliferation (9, 20). EBV-infected B lymphocytes are similar to B lymphocytes proliferating in response to antigen in their growth in tight clumps and expression of B-lymphocyte activation and adhesion molecules (1, 4, 18, 19, 21-24). Nine EBV proteins are expressed in lymphoblastoid cell lines and are the likely mediators of virus-induced B-lymphocyte activation and proliferation (for a review, see reference 11).

In single gene transfer experiments undertaken to evaluate the effects on cell growth of the EBV genes expressed in lymphoblastoid cell lines, a gene which encodes an integral membrane protein, LMP1, had profound effects. When transfected into immortalized rodent fibroblasts, LMP1 had growth transforming effects and caused cells to exhibit loss of contact inhibition, anchorage independence, or tumorigenicity in nude mice (2, 25, 26). In EBV-negative human B-lymphoma cells, which characteristically grow as single cells in culture and express low levels of activation or adhesion markers, LMP1 caused cells to grow in large clumps and up-regulated expression of activation and adhesion molecules (12, 27, 28). These effects on cell growth and activation indicate that LMP1 is an important mediator of EBV effects on lymphocyte growth.

The LMP1 mRNA sequence is predicted to encode a protein which contains six 20-amino-acid, highly hydrophobic, alpha-helical transmembrane domains and intracellular amino and carboxy termini (7, 10; Fig. 1). The results of immunofluorescence staining and live-cell LMP1 protease cleavage studies are consistent with the proposed orientation and also indicate that LMP1 localizes to the plasma mem-

brane of latently infected B lymphocytes (Fig. 1A; 10, 14-16). In B lymphocytes, LMP1 constitutively forms plasma membrane patches and caps which are associated with vimentin intermediate filaments (10, 13, 14). Presumably as a consequence of vimentin cytoskeletal association, 50% of the LMP1 in B lymphocytes or in rodent fibroblasts resists extraction in buffers containing nonionic detergents (13, 16, 17). However, LMP1's ability to form patches and caps in the plasma membrane and to alter the B-lymphocyte phenotype is independent of vimentin association, since LMP1 is phenotypically active in the Daudi B-lymphoma cell line, which lacks any detectable vimentin (12).

The objective of the present study is to more closely examine the functional domains of LMP1 in B-lymphoma cells. Previous studies in B-lymphoma cells with an amino-terminally truncated LMP1 which consisted of only the last two transmembrane domains and the carboxy-terminal cytoplasmic domain revealed a complete lack of functional activity, as judged by its inability to alter cell growth or cell surface expression of adhesion molecules. The mutant protein associated with cytoplasmic membranes, including the plasma membrane, but did not associate with the cytoskeleton or aggregate in plasma membrane patches (27). Since the inactivity of this mutant could be due to the absence of the amino-terminal cytoplasmic domain or to the absence of the first four transmembrane domains, we have extended the genetic analysis by evaluating the effects of deleting the first 16 amino acids of the amino-terminal cytoplasmic domain, the entire amino-terminal cytoplasmic domain and the first transmembrane domain, or the third and fourth transmembrane domains (Fig. 1). These mutant LMP1 proteins were assayed for their effects on B-lymphoma cells and compared in parallel with the effects of LMP1 and the lack of effect of the previously described mutant which encodes a protein consisting of the last two transmembrane and carboxy-terminal cytoplasmic domains.

LMP1 deletion mutants were constructed from an LMP1 cDNA as described in the legend to Fig. 1B. After verification of the constructs by restriction endonuclease digestion,

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FIG. 1. (A) Model of LMP1 orientation in the plasma membrane. The N and C termini are located in the cytoplasm. Potential trypsin (↑) and chymotrypsin (↓) cleavage sites are indicated, and these proteases have previously been used in mapping the orientation of LMP1 in the plasma membrane (12). (B) Schematic illustration of LMP1 and mutant forms of LMP1. The amino and carboxy termini are labeled N and C, respectively. The three pairs of transmembrane domains are labeled 1, 2, and 3. Deletion mutations were made in an LMP1 cDNA which had been cloned in a Bluescript (Stratagene) plasmid (13). The deletion mutants were named for the restriction enzymes used in their construction. LMPΔT was constructed by deleting the first 36 bases of the LMP1 open reading frame 5' to the *Tha*I site and ligating the 5'-truncated cDNA to an oligonucleotide encoding methionine and threonine so that the arginine encoded by the 13th LMP1 codon is the third amino acid. LMPΔS was constructed by deleting the first 75 bases of the LMP1 open reading frame 5' to the *Stu*I site, thereby allowing the methionine encoded by the 44th LMP1 codon to initiate translation. LMPΔBX was made by cutting the cDNA at the *Bcl*I site at nucleotide position 232 and at the *Xho*II site at nucleotide position 400 and religating the compatible ends, thereby rejoining the cDNA in frame. LMPΔX was made by deleting the first 368 bases of the LMP1 open reading frame 5' to the *Xho*II site, thereby allowing the methionine encoded by the 129th LMP1 codon to initiate translation.

in vitro transcription, translation, immunoprecipitation, and gel electrophoresis, the deletion mutants or the wild-type LMP1 cDNAs were cloned into the pSG5 expression vector (Stratagene) under control of the simian virus 40 early promoter. Wild-type or deletion mutant LMP1 cDNAs in pSG5 and a vector for expression of the hygromycin phosphotransferase gene were then transfected into BJAB B-lymphoma cells which have been previously used to assay LMP1 effects (28). Cells (5×10^6) were transfected by electroporation with 50 μ g of pSG5, pSG5 and LMP1, or pSG5 and mutant LMP1 and with 5 μ g of the hygromycin B phosphotransferase expression vector. The cells were electroporated using a proGenetor II electroporator (Hoefer Scientific Instruments, San Francisco, Calif.) set at 1,080 μ F/295 V/1,000 ms. The cells were then fed with fresh RPMI 1640 medium supplemented with 10% fetal calf serum. After 48 h, cells were plated into microtiter wells under selective conditions. For selection, 1,000 cells were plated into each microwell in 200 μ l of complete medium with 400 μ g of hygromycin B (Calbiochem) per ml. Cells were fed every 4 days with fresh selective medium until hygromycin B-resistant clones emerged 2 to 3 weeks after plating.

Clones of resistant cells were expanded and assayed for

LMP1 expression. Five BJAB clones stably expressing LMPAT (amino acids 2 to 12 deleted and replaced by a threonine), LMPΔS (amino acids 1 to 43 deleted) or LMPΔX (amino acids 1 to 128 deleted) in amounts similar to LMP1 in EBV-transformed lymphoblastoid cell lines were derived (Fig. 1 and 2). Despite several attempts, only two BJAB clones which expressed the LMPΔBX mutant in which the third and fourth transmembrane domains have been deleted could be derived (Fig. 1 and 2). Four wild-type LMP1 clones and three vector control clones were derived in parallel (Fig. 2). The sizes of each of the mutant proteins in BJAB cells were the same as those of the proteins translated in vitro from the respective in vitro transcripts (data not shown).

For intracellular localization of LMP1 by immunofluorescence microscopy, cells were washed in phosphate-buffered saline, fixed in absolute methanol for 10 min at -20°C , stained using S12 (anti-LMP1) monoclonal antibody (15) and then a fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Jackson ImmunoResearch, Inc., West Grove, Pa.), and photographed using a Zeiss Axiomat microscope equipped for epifluorescence (13). The protein encoded by each LMP1 deletion mutation had a characteristic intracellular distribution (Fig. 3). As previously re-

FIG. 2. Immunoblot analysis of LMP1- and mutant LMP1-expressing BJAB clones. Expression levels of LMP1 or mutant LMP1 in individual clones are demonstrated by immunoblotting using the S12 (anti-LMP1) monoclonal antibody (15, 27). An equal number of cells was electrophoresed in each lane. Clones expressing LMP1 (lanes 1 to 4), LMP Δ T (lanes 5 to 9), LMP Δ BX (lanes 10 and 11), LMP Δ S (lanes 12 to 16), LMP Δ X (lanes 17 to 21), and vector controls (lanes 22 to 25) are shown. Molecular mass standards of 66 and 43 kDa are indicated.

ported, LMP1 constitutively organized into small patches which formed a large cap in the plasma membrane (12–14, 27). LMP Δ T was similarly localized except that the patches were organized into a cap which is more diffusely distributed in the membrane than LMP1 was (Fig. 3). LMP Δ S, LMP Δ BX, and LMP Δ X differed from LMP1 and LMP Δ T in their intracellular localization. LMP Δ S was distributed in a patchy diffuse rim at the cell periphery compatible with diffuse plasma membrane localization, while LMP Δ BX localized to a diffuse rim probably in the plasma membrane but was also diffusely distributed in the cytoplasm. LMP Δ X was diffusely distributed in the cytoplasm without any peripheral accentuation, as has been described previously (27). Cell membrane fractionation studies indicate that the LMP Δ X fluorescence localization correlates with the presence of LMP Δ X in all cytoplasmic membrane fractions including the plasma membrane (11a, 26, 27).

As in previous studies, LMP1 expression in B-lymphoma cells resulted in homotypic adhesion, as evidenced by growth in tight clumps (10, 27, 28; LMP [Fig. 4]), while LMP Δ X expression did not cause homotypic adhesion (27; Fig. 4). LMP Δ T also caused BJAB cells to grow in tight

FIG. 3. Immunofluorescence staining pattern of LMP1 and mutant forms of LMP1 in BJAB cells. BJAB cells expressing LMP1, mutant forms of LMP1, or vector controls were fixed, stained with S12 (anti-LMP) antibody and fluorescein isothiocyanate-conjugated goat anti-mouse antibody, and photographed as described previously (13).

clumps, and the clumps were almost as large as those caused by wild-type LMP1, while LMP Δ S caused BJAB cells to grow in large loose clumps (Fig. 4). LMP Δ BX caused BJAB cells to grow in small loose clumps (Fig. 4).

In previous studies, LMP1 expression in EBV-negative B-lymphoma cells resulted in decreased cell surface expression of CD10 and increased cell surface expression of CD23, CD39, CD40, CD44, LFA-1, LFA-3, or ICAM-1 (12, 27, 28). These effects varied somewhat among different B-lymphoma cell lines used to assay LMP1 function (28). In contrast to LMP1, LMP Δ X had shown no effect on B-lymphoma cell growth or cell surface expression of CD23, LFA-1, LFA-3, or ICAM-1 (27). The effects of wild-type or mutant forms of LMP1 on the cell surface expression of CD10, CD23, CD39, CD40, CD44, LFA-1, LFA-3, and ICAM-1 in BJAB cells were therefore determined by flow cytometry (Table 1). Neither wild-type nor deletion mutant LMP1 proteins had any effect on BJAB expression of CD44 or LFA-1. In contrast, LMP1 caused significantly decreased cell surface expression of CD10 and increased expression of CD23, CD39, CD40, LFA-3, and ICAM-1 (Table 1). The effect on CD39 was small (Table 1). As in the previous study (27), LMP Δ X had no effect on cell surface molecule expression (Table 1). The newly derived LMP1 mutants had intermediate effects on BJAB surface molecules. LMP Δ T- and LMP Δ S-expressing BJAB clones had decreased levels of CD10 and elevated LFA-3 and ICAM-1 levels. In this regard, they were similar to LMP1-expressing clones (Table 1). However, these clones had little, if any, up-regulated cell

FIG. 4. Morphologic characteristics of LMP1- and mutant LMP1-expressing BJAB cells. Cells were plated at equal densities, allowed to grow undisturbed for 5 days, and then photographed.

surface expression of CD23, CD39, or CD40, in contrast to the LMP1-expressing clones (Table 1). The two stable LMPΔBX-expressing BJAB clones had significantly decreased levels of CD10 and increased levels of CD39 and LFA-3. LMPΔBX did not up-regulate the cell surface expression of CD23, CD40, or ICAM-1 whose expression was up-regulated by LMP1 (Table 1).

The experiments described here are most compatible with the hypothesis that the amino-terminal cytoplasmic, first transmembrane, and middle two transmembrane domains each contribute to LMP1's effects on B lymphocytes. A protein consisting of the last two LMP1 transmembrane domains and the carboxy terminus (LMPΔX) is not only inactive in affecting CD23 expression, cell clumping, or adhesion molecule expression, as was previously noted (27), but is also broadly inactive in inducing cell surface expression of activation molecules or in down-regulating CD10.

Deletion of only the 2nd to the 12th LMP1 amino acids (LMPΔT) had a small but definite effect on activity. The mutant protein still formed tight patches which aggregated into a larger cap in the plasma membrane. The cells grew as tight clumps, and CD10, LFA-3, and ICAM-1 expression were affected to the same extent as they were by LMP1. However, in contrast to LMP1-expressing cells, the plasma membrane caps were not as tightly organized, the cell clumps were smaller, and there was little, if any, effect on CD40 and CD23. Surprisingly, little additional decrease in LMP1 activity was noted with deletion of the rest of the amino-terminal cytoplasmic domain and the first transmembrane domain (LMPΔS). This mutant protein did not form a large cap in the plasma membrane and did not induce tight cell clumping but was as active as the LMPΔT deletion in altering surface molecule expression. Whether the difference in activity of LMPΔS and LMPΔT in affecting cell clumping is due to the deletion of the rest of the amino-terminal cytoplasmic domain (NH₂-M-R-G-P-P-COOH), to the deletion of the first transmembrane domain, or to the effects of the deletion on the membrane positioning of the second transmembrane domain is not resolved by these experiments. The third and fourth transmembrane domains also appear to be essential for much of LMP1's effects. This mutant (LMPΔBX) differed from the LMPΔX null mutant in forming some punctate aggregates at the cell periphery, in causing cells to grow in loose clumps, and in having some effect on CD10, CD39, and LFA-3 expression. However, LMPΔBX differed from LMPΔS, LMPΔT, and LMP1 in having no effect on CD40, CD23, and ICAM-1. The markedly reduced activity of this mutation indicates that at least some of the plasma membrane aggregating properties and effects on cell activation and adhesion of LMP1 are likely to be mediated by or require the third and fourth transmembrane domains. Although LMPΔBX's effects on the cell surface expression were significant by the statistical method described in Materials and Methods, the fact that we were able to generate and analyze only two LMPΔBX clones leaves some potential for inaccuracy in interpreting the effects of this mutation.

The effects of these mutations can and should be assayed in other cells whose growth is affected by LMP1. LMP1 alters epithelial cell growth, changing the morphology of transformed epithelial cell lines in both monolayer and stratified raft cultures (5, 6). Furthermore, LMP1 expression in the skin of transgenic mice induces epidermal hyperplasia and alters keratin gene expression (29). Moreover, LMP1 has transforming effects in immortalized rodent fibroblast

TABLE 1. Cell surface antigen expression in BJAB cells expressing wild-type or mutant forms of CMP

LMP mutant (n)	Mean fluorescent antibody intensity (SD) ^a							
	CD10	CD40	CD23	CD39	CD44	LFA-1	LFA-3	ICAM-1
None (vector control) (3)	5.6 (0.8)	133.8 (17.4)	0.3 (0.2)	2.0 (0.5)	0.2 (0.3)	5.7 (1.3)	36.0 (16.7)	36.5 (4.7)
LMPΔX (5)	6.0* (1.8)	120.6* (15.2)	0.3* (0.4)	0.8* (0.2)	0.2* (0.2)	3.6* (0.8)	42.5* (5.7)	36.1* (9.2)
LMPΔBX (2)	1.8** (0.3)	178.1* (7.8)	0.6* (0.1)	4.7*** (2.8)	0.3* (0.1)	6.4* (0.5)	94.5*** (10.0)	70.0* (5.0)
LMPΔS (5)	0.1** (0.1)	184.9* (32.7)	1.2* (0.7)	1.7* (0.3)	0.7* (0.9)	3.9* (0.6)	110.3*** (14.4)	119.8*** (20.5)
LMPΔT (5)	0.5** (0.3)	174.1* (14.5)	2.0* (0.4)	2.4* (0.7)	0.1* (0.1)	10.0* (1.9)	95.9*** (20.5)	102.8*** (17.2)
LMP1 (4)	0.1** (0.2)	310.3*** (52.2)	9.7*** (5.0)	3.5*** (0.1)	0.2* (0.3)	5.0* (1.3)	96.9*** (9.4)	157.4*** (30.3)

^a For fluorescence-activated cell sorting analysis, live cells were stained with antibodies and quantitatively analyzed on a FACS flow cytometer (Becton Dickinson & Co.). The antibodies have been described previously (28). The mean value of the mean fluorescent antibody intensity of each surface molecule was determined for each group of BJAB clones. The values were then compared with one another using the Student-Newman-Keuls test, which is a multiple-comparison procedure (18). This is essentially a modification of a *t* test which allows multiple step-wise comparisons to be made among samples which contain more than two groups within them. Statistically significant increase (***), decrease (**), or no effect (*) on cell surface antigen expression relative to that of vector control-transfected clones is indicated.

lines such as Rat-1 (25) and BALB/c 3T3 (2, 13, 26), and molecular genetic analyses of LMP1 effects have been undertaken in these assay systems as well (3, 17, 26). As in lymphocytes, the amino-terminally deleted LMPΔX which consists of the last two transmembrane domains and the cytoplasmic carboxy-terminal domains also has no effect on Rat-1 or BALB/c 3T3 cell growth (3, 26). Addition of the 25-amino-acid amino-terminal cytoplasmic domain to the last two transmembrane and cytoplasmic carboxy-terminal domains does not restore LMP1's effect in causing anchorage-independent growth of BALB/c 3T3 cells (17). Thus, these results are consistent with the B-lymphocyte results in indicating a role for some of the first four transmembrane domains in cell growth transformation. Somewhat in contrast to the results in lymphocytes, in BALB/c 3T3 cells, deletion of 25 amino acids from the amino-terminal cytoplasmic domain or of the entire amino-terminal cytoplasmic domain and the first transmembrane domain not only reduced LMP1's effects on cell growth but completely eliminated these effects (3, 26). The partial effect of LMPΔT or LMPΔS which is evident in B-lymphoma cells could reflect a difference in the repertoire of LMP1's biochemical interactions in the human B lymphocyte as opposed to rodent fibroblasts. Further, deletions of the carboxy-terminal cytoplasmic domain of LMP1 appear to not alter LMP1's effect on anchorage-independent growth of BALB/c 3T3 cells. Such mutants have not yet been assayed in B lymphocytes.

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