Preferential Localization of the Epstein-Barr Virus (EBV) Oncoprotein LMP-1 to Nuclei in Human T Cells: Implications for Its Role in the Development of EBV Genome-Positive T-Cell Lymphomas

Jingwu Xu, Ali Ahmad,* and José Menezes*

Laboratory of Immunovirology, Department of Microbiology and Immunology, University of Montreal and Ste-Justine Hospital, Montreal, Quebec, Canada H3T 1C5

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The Epstein-Barr virus (EBV)-encoded latent membrane protein-1 (LMP-1) is thought to play a role in the EBV-induced B-cell transformation and immortalization. EBV has also been implicated in certain human T-cell lymphomas; however, the phenotypic effects of the expression of this oncoprotein in T cells are not known. To learn whether LMP-1 also induces phenotypic changes in T cells, we stably expressed it in human cell lines of T and B lineages and 25 LMP-1-expressing T-cell clones and 7 B-cell clones were examined. Our results show for the first time that, in sharp contrast to B cells, LMP-1 preferentially localizes to nuclei in T cells and does not induce the phenotypic changes in these cells that it induces in B cells, does not associate with TRAF proteins, and does not arrest the cell cycle in the G₂/M phase. A computer-assisted analysis revealed that LMP-1 lacks the canonical nuclear localization signal. Our results suggest that this oncoprotein may not play the same role in the lymphomagenesis of T cells as it does in B cells.

The Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus which has been linked to several benign and malignant lymphoproliferative and autoimmune disorders, e.g., endemic Burkitt’s lymphoma, nonkeratinizing nasopharyngeal carcinoma, Hodgkin’s disease, Sjögren’s syndrome, etc. The virus induces malignant tumors in experimentally infected New World primates (reviewed in reference 42). Primary EBV infections generally occur in childhood, present with mild symptoms, and are usually self limiting. In developed countries, primary infections are usually delayed until adolescence and are the major cause of infectious mononucleosis. Once infected, the host becomes a lifelong carrier (3, 42). In vitro, EBV can infect resting human B cells and immortalizes them into continuously growing lymphoblastoid cell lines (LCLs) (reviewed in reference 26). These cells express a restricted repertoire of viral genes which include six nuclear antigens (reviewed in reference 26). These cells express a restricted repertoire of viral genes which include six nuclear antigens (reviewed in reference 26).

EBV genome-carrying T-cell lymphomas have been described previously (5, 6, 16, 19, 24, 31, 44, 46). The viral genome contained in these lymphoma cells is monoclonal, which suggests the occurrence of the infection before malignancy and its potential role in the lymphomagenesis. Despite this ability of EBV to infect cells of T lineage, the phenotypic expression of the EBV-encoded antigens in these cells has not been studied. We conducted this study to find out whether the...
EBV oncoprotein LMP-1 induces phenotypic changes in T cells as it does in B cells. To this end, we developed human T- and B-cell clones with stable expression of the transfected LMP-1 gene.

Nuclear localization of LMP-1 in T cells. Initially, we introduced LMP-1 into the EBV genome-negative BJA-B cells (a B-cell line) (32) and CEM cells (a T-cell line) by electroporating (1) a PGEM2-based expression plasmid pIgLMP-1 (kindly provided by Nancy Raab-Traub, University of North Carolina, Chapel Hill) as described earlier (54). The control plasmid was derived from pIgLMP-1 after deleting the LMP-1 sequences. After electroporation, the transfected cells were cultured in the selection medium, cloned by limiting dilutions, and screened for expression of LMP-1 by indirect immunofluorescence. After fixation in methanol, cell smears on slides were first incubated with S12 and CS1-4 anti-LMP monoclonal antibodies, washed, and treated with anti-mouse immunoglobulin G (IgG) fluorescein isothiocyanate-conjugated antibodies. The slides were then examined under an immunofluorescence microscope (54). In sharp contrast to the B (i.e., BJA-B) cell clones, in which LMP-1 was localized to both the plasma membrane and cytoplasm of the cells, in all T (i.e., CEM) cell clones the location of LMP-1 was nuclear only (Fig. 1). In order to determine whether the nuclear localization of LMP-1 was specific to CEM, we expressed LMP-1 in two other T-cell lines, Jurkat and Molt-4, under similar conditions and examined the transfected cells for LMP-1 localization without cloning. As shown in Fig. 2, LMP-expressing Molt-4 cells initially showed three patterns: aggregated patches in the membrane (Fig. 2A), diffused expression in the nuclei (Fig. 2C), and a mixture of these two forms (Fig. 2B). However, an overwhelming majority of the positive cells (>95%) showed nuclear localization. Furthermore, with passage of time, the cells with membrane expression declined and disappeared. The same was observed for LMP-1 expression in Jurkat cells (data not shown). Taken together, these results clearly indicated that although LMP-1 is initially also expressed on the membrane of a few T cells, subsequently, its expression occurs solely in the nuclei of these T cells.

LMP-1 protein expressed in the transfectants has a normal molecular mass (i.e., 63 kDa). The third exon of the LMP-1 gene contains an initiation site, and an amino terminus-deleted form of LMP-1 (45 kDa) occurs naturally in EBV-infected B cells (18, 49). This truncated form of LMP-1 does not form aggregates in plasma membranes. To determine whether the unusual localization of LMP-1 in T cells was due to the preferential expression of this naturally truncated form of LMP-1 in these cells, we performed Western blotting for this protein.
on whole-cell lysates. Cells were lysed in 50 mM Tris-HCl (pH 6.8) and 2% sodium dodecyl sulfate (SDS) and sonicated. After clarification by centrifugation, 40 μg of the lysate protein was resolved by SDS–10% polyacrylamide gel electrophoresis (PAGE) and then transferred onto a nylon membrane. The resolved proteins on the blots were incubated with S12 antibodies and detected with alkaline phosphatase-conjugated secondary antibodies (54). As shown in Fig. 3A, the electrophoretic mobility of LMP-1 in T cells was comparable to that of B cells, suggesting that this protein is not truncated in T cells. To further confirm the localization of LMP-1 in T and B cells, we selected three LMP-1-expressing clones from each cell line (BJA-B and CEM) for further analyses of their cytoplasmic and nuclear fractions (20). These cloned cells were washed with phosphate-buffered saline and resuspended in 200 μl of the lysis buffer (10 mM Tris-HCl [pH 7.5], 150 mM KCl, 3 mM MgCl₂, 1 mM CaCl₂, 2% 90-kDa polyvinylpyrrolidone, 0.2% bovine serum albumin, and 0.5% Nonidet P-40). After vigorous shaking, the resuspension was gently overlaid on top of 1 ml of the same buffer without Nonidet P-40 to which sucrose was added to a final concentration of 24%. The nuclei were pelleted and resuspended in a buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, and 1.5 mM MgCl₂. Both the nuclear and cytoplasmic fractions were mixed with sample loading buffer, boiled, and resolved on SDS–10% PAGE for Western blotting (54). As shown in Fig. 3B and C, the nuclear fraction in CEM clones and the cytoplasmic fraction in B-cell clones were positive for LMP-1. These data confirm that, unlike in B cells, in T cells LMP-1 localizes in the nuclei.

LMP-mediated effects on transfected cells. To determine whether differential localization of LMP-1 in T and B cells induced differential effects on the cell cycle, cells were incubated for 12 h in serum-free medium to synchronize them for the cell cycle. Then they were maintained in the culture medium for 24 h. One million cells were washed with phosphate-buffered saline and resuspended in 1 ml of buffer containing sodium citrate (1 mg/ml, pH 7.4), RNase (0.05 μg/ml), Igepal CA-630 (3 μl/ml), and propidium iodide (PI; 50 μg/ml). After a 30-min incubation at room temperature, cell cycles were analyzed from cellular DNA content by using Cellfit software. The LMP-expressing BJA-B, but not CEM, cells showed a significantly increased number of cells accumulated in the G2/M phase (Table 1 and Fig. 4). To further demonstrate the effects of LMP-1 on the cell cycle, we performed proliferation assays on pIgLMP-1- and pIgNEO-transfected cells. Cells (10⁵ per well) were cultured in triplicate in RPMI 1640 supplemented with 10% fetal bovine serum and 500 μg of G418/ml for 24 h and then pulsed with 1 μCi of [³H]thymidine ([³H]TdR)/well for 16 h. The radioactivity from harvested cells was measured in a liquid scintillation counter. As shown in Fig. 5, LMP-1 significantly inhibited the rate of [³H]TdR uptake by BJA-B cells (P < 0.01) but not by CEM cells (P > 0.05). Clearly, the cell cycle arrest in the G2/M phase induced by LMP-1 resulted in the prolongation of the cell cycle and the

FIG. 2. Different patterns of LMP-1 localization in Molt4 cells. LMP-expressing Molt4 cells were stained with S12 as described in the legend to Fig. 1. Shown here are three different patterns of LMP-1 localization in these cells: on the membrane (A), both on the membrane and in the nucleus (B), and in the nucleus (C). Panel D represents an LMP-expressing cell stained with a control (negative) antibody.

FIG. 3. Detection of LMP-1 by Western blotting in CEM and BJA-B cells. (A) Whole-cell lysates (40 μg) from LMP-expressing and control vector-transfected cells were resolved by SDS–10% PAGE. After transfer onto nylon membranes, LMP-1 was detected by anti-LMP monoclonal antibody S12 followed by detection with alkaline phosphatase-conjugated anti-mouse antibodies 5-bromo-4-chloro-3-indolyolphosphate (BCIP) and nitroblue tetrazolium. Lanes: 1, BJA-B transfected with the control vector; 2 to 4, three different LMP-expressing BJA-B clones; 5, CEM transfected with the control vector; 6 to 8, three different LMP-expressing CEM clones; 9, an EBV-transformed B-cell line (LCL). The LMP-expressing BJA-B and CEM cells were fractionated into cytoplasmic and nuclear lysates, and each fraction was analyzed for LMP-1 expression by Western blotting. Panels B and C show LMP-1 expression in cytoplasmic and nuclear fractions, respectively. The lanes represent the clones indicated for panel A. The arrows point to LMP-1 bands.
LMP-expressing CEM cells, in the G2/M phase. Note the accumulation of LMP-expressing BJA-B cells, but not of

Inhibition of proliferation of BJA-B cells. These data strongly suggest that LMP-1 expressed in the nuclei of CEM cells does not affect their normal progression through the cell cycle as it does in B cells.

LMP-1 does not interact with TRAF-1, nor does it induce Bcl-2, LFA-1, and ICAM-1 expression in T cells. In B cells, LMP-1 is known to interact with TRAFs and induce the expression of the antiapoptotic protein Bcl-2 (2, 9). In order to see if intranuclear LMP-1 was also able to exert these effects in T cells, we first performed Western blotting to analyze the level of TRAF1 protein in LMP-1-expressing CEM and BJA-B cells. As shown in Fig. 5A, LMP-1 significantly induced TRAF1 expression in BJA-B cells but not in CEM cells. Further, we immunoprecipitated TRAF1 from the cytoplasmic fractions and whole-cell lysates of LMP-expressing CEM and BJA-B cells and determined whether LMP-1 interacts with TRAF1 protein. As shown in Fig. 5B, LMP-1 coimmunoprecipitated with TRAF1 in both cytoplasmic fractions and whole-cell lysates of B cells and not of T cells. Similarly, LMP-1 induced the expression of Bcl-2, LFA-1, and ICAM-1 in B cells but not in T cells (Fig. 6).

LMP-1 does not induce a tyrosine-phosphorylated protein in T cells. LMP-1 is known to be phosphorylated in B cells at serine and threonine residues, and this phosphorylation is necessary for its association with the cytoskeleton and patching in the plasma membranes of these cells (30, 34). To determine whether LMP-1 was also phosphorylated on tyrosine residues in T cells, we performed comparative analysis with Far Western blots in which we immunoprecipitated LMP-1 from T and B cells and determined its reactivity with phosphotyrosine-specific antibody (54). Consistent with a previous report (34), LMP-1 was not tyrosine-phosphorylated in either T or B cells (data not shown). We further determined whether LMP-1 expression in these cells induced tyrosine phosphorylation on any protein. Therefore, Western blots prepared from the LMP-expressing and control cells were probed with anti-phosphotyrosine antibodies. As shown in Fig. 7, LMP-1 expressed in BJA-B cells (but not in CEM cells) induced a protein that reacted very strongly with phosphotyrosine-specific antibodies and migrated to approximately 40 kDa on these blots. The identity of this protein remains unknown. Interestingly, the anti-phosphotyrosine-reactive LMP-induced protein migrated to a lower level in EBV-transformed (control) LCLs, which may be due to its differential posttranslational processing.

We have demonstrated here that the EBV oncoprotein LMP-1 predominantly localizes in the nuclei upon its forced expression in human T-cell lines. This is in contrast to its expression in B cells, in which it occurs as aggregated patches in the plasma membranes. It is by virtue of its ability to become aggregated in the plasma membrane that LMP-1 acts as a constitutively activated receptor of the TNF receptor superfamily, particularly CD40 (10, 48). Three different regions in the intracellular cytoplasmic terminus of LMP-1 interact with various TRAFs, transduce intracellular signals, and bring about the phenotypic changes seen in the EBV-immortalized B cells. Six hydrophobic membrane-spanning regions of the LMP-1 molecule impart to it the ability to form aggregated patches in the plasma membrane (9). A naturally occurring truncated version of LMP (45 kDa) is expressed from the EDL 1 promoter late in the lytic cycle (11). It has only two hydro-

### Table 1. Effects of LMP-1 expression on cell cycle progression

<table>
<thead>
<tr>
<th>Cell line</th>
<th>% of cells in cell cycle stage</th>
<th>$G_0/G_1$</th>
<th>$S$</th>
<th>$G_2/M$</th>
<th>$G_2/G_1$ ratio</th>
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<tbody>
<tr>
<td>BJA-B IgNEO</td>
<td>29.26</td>
<td>66.52</td>
<td>3.86</td>
<td>1.80</td>
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<tr>
<td>BJA-B IgLMP-1(A)</td>
<td>34.39</td>
<td>46.91</td>
<td>18.70</td>
<td>1.86</td>
<td></td>
</tr>
<tr>
<td>BJA-B IgLMP-1(B)</td>
<td>32.51</td>
<td>48.81</td>
<td>18.68</td>
<td>1.85</td>
<td></td>
</tr>
<tr>
<td>BJA-B IgLMP-1(C)</td>
<td>39.36</td>
<td>47.41</td>
<td>12.63</td>
<td>1.87</td>
<td></td>
</tr>
<tr>
<td>CEM IgNEO</td>
<td>55.46</td>
<td>42.75</td>
<td>1.80</td>
<td>1.90</td>
<td></td>
</tr>
<tr>
<td>CEM IgLMP-1(A)</td>
<td>51.41</td>
<td>47.94</td>
<td>0.65</td>
<td>1.91</td>
<td></td>
</tr>
<tr>
<td>CEM IgLMP-1(B)</td>
<td>59.87</td>
<td>36.74</td>
<td>3.40</td>
<td>1.81</td>
<td></td>
</tr>
<tr>
<td>CEM IgLMP-1(C)</td>
<td>51.57</td>
<td>45.26</td>
<td>3.17</td>
<td>1.87</td>
<td></td>
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</table>

$^a$ Cell cycles were synchronized by incubating cells for 12 h in serum-free medium and then for 24 h in culture medium before analysis. Cells were analyzed for cell cycle stage after staining with PI by using a flow cytometer and Cellfit software. Note the accumulation of LMP-expressing BJA-B cells, but not of LMP-expressing CEM cells, in the G2/M phase.

![Figure 4](http://jvi.asm.org/)

**FIG. 4.** Cell cycle profiles of LMP-expressing cells. After staining with PI, the cell cycle profile was analyzed from the DNA content by using a flow cytometer and Cellfit software. (A) The figure shows the relative number of cells in various phases of the cell cycle for control vector-transfected BJA-B (a), LMP-expressing BJA-B (b), control vector-transfected CEM (c), and LMP-expressing CEM (d) cells. Note the accumulation of cells in the G2/M phase in LMP-expressing BJA-B cells (b). (B) Proliferation of pIgLMP-1 and control vector (IgNEO)-transfected cells. Cells (105 per well) were cultured in triplicate for 24 h and pulsed with [3H]TdR for 16 h to assess proliferation. The asterisk indicates significant difference ($P < 0.01$).
phobic membrane-spanning regions, and it does not aggregate within the plasma membrane, transform rodent fibroblasts, or induce phenotypic changes characteristic of the EBV-immortalized B cells (11, 18, 49). From the evidence shown above, the LMP-1 expressed in T cells is the authentic full-length (63 kDa) protein and not the truncated version. Furthermore, we have used the same constructs for the expression of LMP-1 in both T and B cells, and it is highly unlikely that mutations in the expression plasmids are responsible for its differential expression features in T and B cells. Given the fact that LMP-1 does not form aggregates at the T-cell plasma membrane, it is not surprising that it does not interact with TRAFs and induce phenotypic changes as seen in the B cells. Unlike B cells, which grow in tight clumps upon LMP-1 expression, LMP-expressing T cells did not clump together (data not shown). Few investigators have reported the expression of LMP-1 in T cells (22, 40). However, either they expressed LMP-1 transiently or under inducible promoters; in any case, they did not look for the location of LMP-1 in their target cells. It is also noteworthy that LMP-1 is rarely detected in the EBV genome-containing peripheral T-cell lymphomas despite its expression of its mRNA (45). Furthermore, immature human thymocytes and a human thymocytic cell line (HPB-ALL), which can be readily infected in vitro with EBV, fail to express LMP-1 (37, 52).

For importation into the nucleus, macromolecules of >50 kDa must be actively translocated across the nuclear pore complex (reviewed in references 22 and 39). LMP-1, being 63 kDa, cannot passively diffuse from the cytoplasm into the nucleus through the nuclear pore complex. The first step in this nuclear import process is the recognition and binding of importin α (or karyopherin α) to the so-called nuclear localization signal (NLS) of the protein (36). It is known that NLS contributes to shuttle protein from the cytoplasm to the nucleus as has been shown, for example, for the simian virus 40 T antigen, nucleoplasmin, and c-myc (10). The canonical NLS is comprised of one (monopartite) or two (bipartite) clusters of conserved basic amino acids. To determine whether the NLS may play a role in LMP-1 localization in the nuclei, we searched for the presence of canonical mono- and bipartite NLSs (PKKKRKV, KRPAATKKAGQAKKK, and PAAKRV KLD) (10) by using the BCM search launcher. LMP-1 was found to be negative for the presence of such a prototypical

FIG. 5. (A) LMP-1 induced the TRAF1 protein (arrow) in BJA-B cells but not in CEM cells. Whole-cell lysates (40 μg) from LMP-expressing and control vector-transfected cells were resolved by SDS–10% PAGE. After transfer onto nylon membranes, TRAF1 was incubated with anti-TRAF1 antibody followed by detection with alkaline phosphatase-conjugated anti-rabbit antibody. The lanes represent different clones (the same as lanes 1 to 9 of Fig. 3). (B) LMP-1 (arrows) interacted with TRAF1 in BJA-B cells but not in CEM cells. TRAF1 was immunoprecipitated from cytoplasmic fractions (a) and whole-cell lysates (b), resolved by SDS-PAGE, and electroblotted onto nylon membranes. The membranes were probed for LMP-1 with LMP-specific monoclonal antibodies (CS1-4) and alkaline phosphatase-conjugated secondary antibodies. Lane 1 represents immunoprecipitation with normal rabbit serum. Lanes 2 to 10 in panel B correspond to lanes 1 to 9 in panel A, respectively.

FIG. 6. Expression of bcl-2 (A), LFA-1 (B), and ICAM-1 (C). LMP expression induces bcl-2, LFA-1, and ICAM-1 in BJA-B cells but not in CEM cells. The expression of these proteins in different clones of LMP-expressing cells was determined by Western blotting performed on 40 μg of whole-cell lysates. The lanes represent the cell clones indicated in the legend to Fig. 3. Arrows from top to bottom indicate bcl-2, LFA-1, and ICAM-1, respectively.

FIG. 7. LMP-1 expression induces a tyrosine-phosphorylated protein in B, but not in T, cells (arrow). Far Western blots were prepared from whole-cell lysates by using immunoprecipitated LMP-1. The blots were probed with anti-phosphotyrosine and alkaline phosphatase-conjugated anti-mouse IgG as described in the legend to Fig. 3. The lanes indicate the cell clones described in the legend to Fig. 3.
NLS when the six frame translation products of its cDNA sequence were searched (data not shown). However, this does not rule out the possibility of the nuclear import of LMP-1 by importin α as the latter protein may interact with a cargo protein in an NLS-independent fashion (41). Furthermore, the consensus sequence for the NLS is now considered to be much broader than has been hitherto recognized (53). Thus, LMP-1 may possess such a noncanonical NLS. In any event, a noncanonical NLS-based nuclear import of LMP-1 in T cells would imply that this NLS may be masked in B cells, probably due to differential posttranslational modifications.

It has been shown that LMP-1 associates with vimentin, which constitutes intermediate filaments of the cytoskeleton and connects the plasma membrane to the nuclear membrane (4, 29). LMP-1 expression causes vimentin to colocalize into patches in the plasma membrane and hence leads to abnormality in the arrangement of the cytoskeleton. The treatment of cells with a vimentin-depolymerizing agent causes vimentin to aggregate as patches in the perinuclear region (6). If these cells are also expressing LMP-1, it colocalizes into the perinuclear region along with vimentin (29). Cytoskeleton-associated proteins (e.g., Vif of human immunodeficiency virus type 1) are known to be nucleophilic as they glide along these filaments into the nucleus (21). Such proteins help transport viral cores to the nuclei of the infected cells. The human T cells used in this study are known to express vimentin, which may be playing a role in the nuclear localization of LMP-1 in these cells.

LMP-1 is expressed both in the viral lytic and latent cycles. It is also incorporated into virions. The protein is essential for in vitro immortalization of B cells and induces phenotypic changes in these cells which are characteristic of their immortalized phenotype. It imparts resistance to apoptosis following serum withdrawal, p53 activation, and the growth inhibitory effects of TGF-β (2, 13). Furthermore, it also inhibits differentiation of epithelial cells (7). Thus, LMP-1 acts as a classical oncoprotein and has been considered to play a role in the development of EBV-associated malignancies. On the other hand, LMP-1 is also cytotoxic and immunogenic (12, 15, 25) and there is no direct proof for its role in the in vivo process of lymphomagenesis. The transgenic mice which express LMP-1 in their B cells under the Ig promoter, however, develop tumors when they are 12 months of age or older (27). The present data would suggest that LMP-1 may not be contributing to the development of EBV genome-containing T-cell lymphomas. A similar conclusion was drawn earlier based upon the rarity of the detection of the LMP-1 protein in peripheral T-cell lymphomas (38, 45). Unfortunately, we were unable to find or obtain cells of T-cell lymphomas for our present study. In any event, the present data demonstrate novel features of LMP-1 expression which appear to be target cell dependent. Chief among these are (i) noninduction of certain cellular changes that are found associated with LMP-1 expression in B cells and (ii) preferential localization of LMP-1 in the nuclei of T cells.

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REFERENCES


