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Epstein-Barr Virus Latent Membrane Protein: Induction of B-Cell Activation Antigens and Membrane Patch Formation Does Not Require Vimentin

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The latent membrane protein (LMP) of Epstein-Barr virus (EBV) forms patches associated with the vimentin intermediate filament system in EBV-transformed lymphoblastoid cell lines, EBV-infected Burkitt's lymphoma cells, and LMP-transfected, EBV-negative Burkitt's lymphoma cells. By gene transfer, LMP induces the expression of vimentin and B-cell activation antigens in EBV-negative Burkitt's lymphoma cells. We have now expressed LMP in an EBV-positive Burkitt's lymphoma cell line, Daudi, which does not express any LMP or vimentin. In these Daudi transfectants, LMP still formed plasma membrane patches in the absence of vimentin. LMP did not resist nonionic detergent extraction in Daudi cells as it does in vimentin-expressing cells. LMP still retained functional activity as judged by induction of B-cell activation antigens. These data indicate that LMP can form plasma membrane patches and induce B-lymphocyte activation independent of vimentin association.

Epstein-Barr virus (EBV) is a prevalent human herpesvirus associated with the etiology of B-cell lymphomas in immunosuppressed patients and African children (for reviews, see reference 3 and 4). EBV infection of B lymphocytes results in continual cell proliferation. Soon after EBV infection of normal B lymphocytes there is a rapid increase in cell size, increased expression of several B-cell activation markers, increased DNA and RNA synthesis, and immunoglobulin secretion (1, 5, 6, 8, 13, 14). A restriction set of viral genes which encode several nuclear proteins and one integral membrane protein (latent membrane protein [LMP]) is expressed in these latently infected, growth-transformed lymphocytes (for a review, see reference 4).

Many of the changes in cellular phenotype which occur upon EBV-induced growth transformation of normal B lymphocytes are reproduced by EBV infection of EBV-negative Burkitt's tumor cell lines. The infected cells express the latent viral genes, grow in tight clumps, and have increased surface expression of B-cell activation markers (2, 12). LMP is likely to be important in inducing lymphocyte proliferation, since in transfection experiments in EBV-negative B-cell lines LMP causes cells to grow in tight clumps and increases surface expression of B-cell activation markers (17). In addition, LMP is able to transform rodent fibroblasts (15, 16). One of the nuclear proteins (EBNA-2) is also likely to be important in EBV-induced lymphocyte proliferation, since it induces the B-cell activation marker CD23 in these same B-cell lines (18).

On the basis of its DNA sequence, LMP is predicted to contain a short hydrophilic amino terminus, followed by six hydrophobic alpha-helical potential transmembrane domains separated by five short reverse-turn peptides, and a long hydrophilic carboxy terminus. In support of this model, live-cell proteolysis experiments indicate a cytoplasmic orientation for the amino and carboxy termini along with two reverse-turn domains and suggest an orientation outside the membrane for the first reverse-turn domain (10). These live-cell proteolysis studies also indicate that at least 30% of the LMP in EBV-transformed lymphocytes is in the plasma membrane. In immunofluorescence microscopy studies, LMP colocalizes to patches at the cell periphery with vimentin intermediate filaments (9). In fact, vimentin can determine the localization of LMP in the cell, since colcemid treatment causes vimentin to reorganize into perinuclear rings and LMP colocalizes to these rings. Further evidence for the cytoskeletal association of LMP is that despite full solubility in isotonic buffers containing nonionic detergent, only 50% of the LMP in EBV-transformed lymphocytes is extracted under these conditions (9). The remaining LMP is bound to the cytoskeleton, presumably either directly or indirectly to vimentin. Time course metabolic labeling studies indicate that shortly after synthesis LMP is phosphorylated and becomes bound to the cytoskeleton (9). Surprisingly, most non-EBV-infected Burkitt's lymphoma cell lines express small amounts of vimentin, and EBV infection or LMP expression causes higher-level vimentin expression.

The importance of the cytoskeletal and plasma membrane associations of LMP is also suggested by transfection studies involving a naturally occurring truncated form of LMP (D1LMP) which is expressed late in viral replication. In contrast to LMP, D1LMP lacks the ability to induce B-lymphocyte activation markers in B-cell lines and does not transform rodent fibroblasts. D1LMP, unlike LMP, is fully extracted from fibroblasts or lymphocytes by isotonic nonionic detergent buffers, indicating that it does not associate with the cell cytoskeleton (16, 17). Furthermore, unlike LMP, the D1LMP in the B-cell lines does not form patches or localize to the cell periphery.

These accumulated data are consistent with the working hypothesis that LMP function in cell transformation or activation requires plasma membrane patches and association with the cytoskeleton, possibly specifically with vimentin. In the course of recent studies establishing a role for LMP in inducing higher-level vimentin expression in EBV-negative Burkitt's tumor cells, we came across an EBV-infected Burkitt's tumor cell line, Daudi, which contains no detectable vimentin at the level of mRNA or protein expres-

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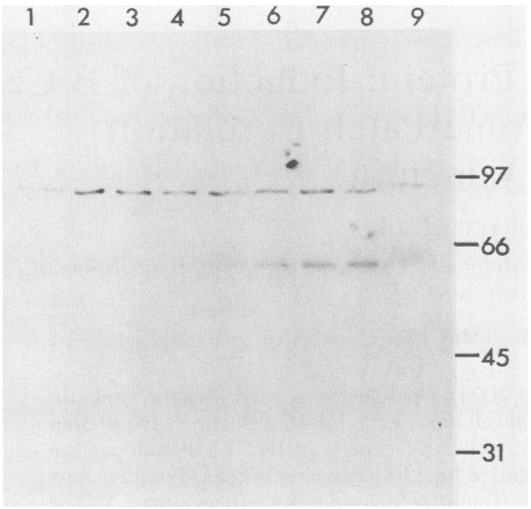

FIG. 1. LMP expression in Daudi-LMP transfectants. A total of 2×10^5 cells were solubilized in sodium dodecyl sulfate sample buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting with S12 (anti-LMP) monoclonal antibody as previously described (10). Lanes: 1, Louckes cells; 2, Daudi cells (untransfected); 3, Daudi-gpt c.4; 4, Daudi-gpt c.5; 5, Daudi-LMP c.2; 6, Daudi-LMP c.3; 7, Daudi-LMP c.6; 8, Daudi-LMP c.17; 9, IB-4. The molecular weights (in thousands) of protein standards are indicated on the right.

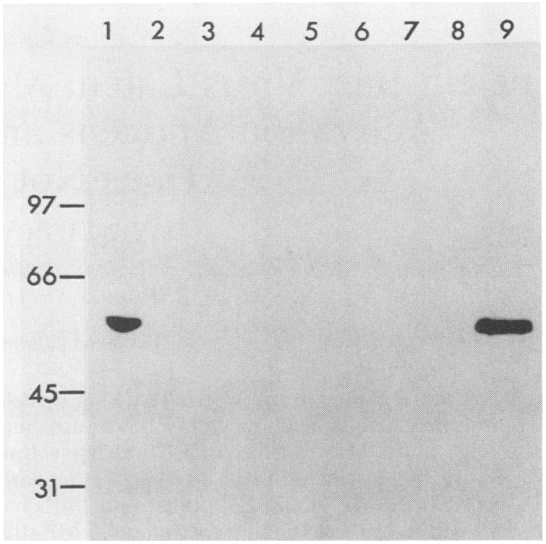

FIG. 2. Vimentin expression in Daudi-LMP transfectants. A total of 2×10^5 cells were solubilized in sodium dodecyl sulfate sample buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting with an antivimentin monoclonal antibody as previously described (9). Lanes: 1, Louckes cells; 2, Daudi cells (untransfected); 3, Daudi-gpt c.4; 4, Daudi-gpt c.5; 5, Daudi-LMP c.2; 6, Daudi-LMP c.3; 7, Daudi-LMP c.6; 8, Daudi-LMP c.17; 9, IB-4. The molecular weights (in thousands) of protein standards are indicated on the left.

sion and no LMP. In addition, Daudi does not express EBNA-2 due to a deletion in the viral genome (7). Daudi is well suited for studies of the role of vimentin in LMP function and patch formation since it does not already express B-cell activation markers normally associated with EBV infection.

Initial screening of vimentin expression in B-cell lines was performed by immunoblotting and indirect immunofluorescence staining with a monoclonal antibody to vimentin. The EBV-negative Burkitt's lymphoma cell lines BL-30, BL-41, and Louckes contained detectable levels of vimentin. The Burkitt's lymphoma cell line Daudi, along with several other Burkitt's lymphoma cell lines, had previously been reported not to express any detectable vimentin mRNA (11). However, when we screened many of the same cell lines, vimentin was detected by immunoblotting (9). Daudi is the only Burkitt's lymphoma cell line we have screened which contains no detectable vimentin in immunoblotting assays.

The LMP gene was introduced into Daudi cells under control of the human metallothionein type 2 promoter by electroporation in a vector which allowed the cells to be placed under positive selection (15–17). Vector-transfected control and LMP-transfected cell clones emerged from 96-well plates after approximately 20 days of selection. The vector control cells grew in small chains of 5 to 10 cells that were indistinguishable in growth from untransfected Daudi cells. In contrast, the LMP-transfected Daudi cells grew as tight clumps of 50 to 200 cells. Different clones of LMP-transfected Daudi cells varied in their characteristic clump size.

The levels of LMP expression in four representative LMP-transfected Daudi clones, two vector-transfected Daudi clones, and untransfected Daudi cells were checked by Western immunoblot. The four LMP-transfected Daudi clones, Daudi-LMP c.2, c.3, c.6, and c.17 (Fig. 1, lanes 5, 6, 7, and 8, respectively), expressed levels of LMP similar to those expressed by the EBV-transformed lymphoblastoid cell line IB-4 (Fig. 1, lane 9). As expected, untransfected Daudi cells (Fig. 1, lane 2) and two vector-control transfected Daudi clones, Daudi-gpt c.4 and c.5 (Fig. 1, lanes 3 and 4, respectively) expressed no detectable LMP, as did Louckes, an EBV-negative Burkitt's tumor cell line (Fig. 1, lane 1).

Vimentin expression in these cells was determined by Western immunoblot. As expected, Louckes and IB-4 cells (Fig. 2, lanes 1 and 9, respectively) expressed detectable levels of vimentin, while Daudi cells and all of the vector control and LMP-expressing lines derived from them (Fig. 2, lanes 2 through 8) did not.

Previously, LMP has been shown to induce surface expression of several B-cell activation antigens, such as the cell adhesion molecules LFA-1, ICAM-1, and LFA-3, in the EBV-negative cell line Louckes (17). Daudi clones expressing LMP exhibited increased cell surface expression of LFA-1, LFA-3, and ICAM-1 compared with untransfected or vector-transfected Daudi clones as determined by quantitative flow cytometry (Table 1). The increased surface expression of lymphocyte adhesion molecules in Daudi-LMP cells indicates that LMP does not require vimentin to induce these B-cell activation markers.

We have previously shown that 50% of the total cellular LMP in EBV-transformed lymphoblastoid cell lines and Burkitt's tumor cells resists extraction in phosphate-buffered saline containing 0.5% Triton X-100 (PBS–TX-100 [9]). This finding was extended to EBV-negative Burkitt's tumor cells and rodent fibroblasts into which LMP was transfected (16, 17). In addition, extraction of EBV-positive cells with high-salt (0.6 M) buffer containing 0.5% Triton X-100 (PBHS–TX-100) releases 65% of the total cellular LMP, with 35% resisting extraction (9). The origin of the detergent-insoluble LMP was proposed to be due to its association with vimen-

TABLE 1. Cell surface adhesion molecule expression in Daudi-LMP transfectants

Clone	Fluorescence[a]			
	BKG	LFA-1	LFA-3	ICAM-1
Untransfected parental Daudi line	0.2	1.0	1.3	53
Daudi-gpt c.4	0.3	1.2	0.9	51
Daudi-gpt c.5	0.3	0.9	1.6	62
Daudi-LMP c.2	0.2	22	20	152
Daudi-LMP c.3	0.4	29	32	167
Daudi-LMP c.6	0.2	17	18	143
Daudi-LMP c.17	0.3	24	25	155

[a] Mean fluorescence intensity (from fluorescent-antibody staining) normalized for cell surface area. Numbers greater than 5 were rounded to nearest whole number. BKG, background staining.

tin, since LMP and vimentin are colocalized in these cells as determined by double immunofluorescence staining.

If the presence of a detergent-insoluble LMP fraction is due to its association with vimentin, then much more of the total LMP present in Daudi cells should partition to the detergent-soluble fraction than in previously studied cell types. Daudi-LMP c.3 was extracted three times in PBS–TX-100. The amount of LMP in the three extracts and in the detergent-insoluble residue was determined by gel scanning densitometry of Western immunoblots. Greater than 90% of the total cellular LMP in Daudi-LMP c-3 was extracted in PBS–TX-100 (Fig. 3, lane 1), with 6 to 8% remaining insoluble (Fig. 3, lane 4). The same result was obtained for Daudi-LMP c.17 (data not shown). This result is very

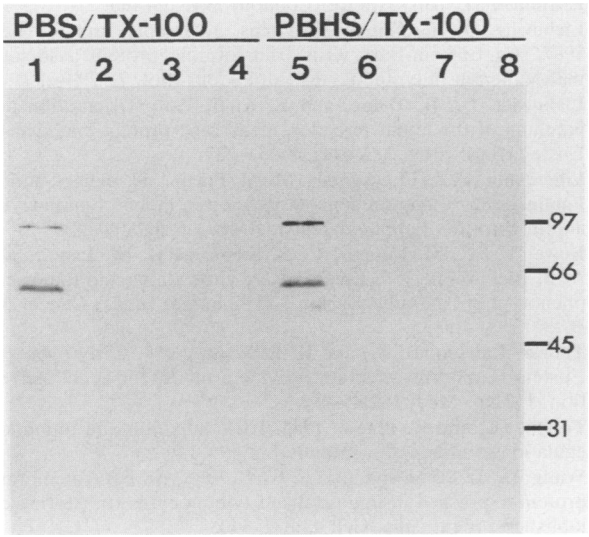

FIG. 3. Detection of LMP in detergent-soluble and detergent-insoluble fractions of Daudi-LMP cells. Cells were extracted three times with PBS–TX-100 (physiological salt concentration) (lanes 1 to 4) or PBHS–TX-100 (high salt concentration) (lanes 5 to 8) (9). The fractions were solubilized in sodium dodecyl sulfate sample buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting with S12 (anti-LMP) monoclonal antibody. Lanes: 1 and 5, protein samples from first detergent extraction; 2 and 6, protein samples from second detergent extraction; 3 and 7, protein samples from third detergent extraction; 4 and 8, detergent-insoluble cell residue. An equal percentage of each fraction corresponding to 10^6 cells was loaded in each lane. The molecular weights (in thousands) of protein standards are indicated on the right.

different than that obtained when LMP-transfected Louckes cell lines were extracted in the same manner. In Louckes cells, only 50% of the LMP is extracted, while 50% remains insoluble (16). The proportions reported here represent the results of several scans for four separate extractions performed with each cell line.

When Daudi-LMP cells were extracted in PBHS–TX-100, greater than 95% of the LMP was extracted (Fig. 3, lane 5), with 2 to 5% remaining insoluble (Fig. 3, lane 8). In contrast, when IB-4 or Louckes-LMP cells are extracted with PBHS–TX-100, 65% of the LMP is extracted and 35% resists extraction (9; D. Liebowitz, unpublished observation).

Therefore, a much greater proportion of the LMP in Daudi cells is extracted in PBS–TX-100 and PBHS–TX-100 than is extracted from other cell types. This is consistent with the hypothesis that the majority of the detergent-insoluble LMP arises due to association with vimentin.

If LMP patching requires vimentin association, then the findings that Daudi cells contain no vimentin and that LMP is nearly completely extracted by nonionic detergent buffers would suggest that LMP may not be patched in Daudi cells. However, indirect immunofluorescence staining of fixed cells revealed that LMP was tightly patched and capped in Daudi-LMP clones (Fig. 4B and C). As expected, vector control Daudi-gpt clones did not stain for LMP (Fig. 4A), and IB-4 cells had patches of LMP (data not shown). Staining of fixed cells for surface immunoglobulin M showed diffuse plasma membrane fluorescence which was not patched in appearance (data not shown). Therefore, LMP patching does not require vimentin association, and detergent-soluble LMP forms patches in B lymphocytes.

These experiments demonstrate that LMP can alter the B-lymphocyte phenotype and form patches without interacting with the vimentin cytoskeletal network. Importantly, the lack of a detergent-insoluble form of LMP in Daudi-LMP cells supports the hypothesis that this form of LMP is bound to vimentin, while the detergent-soluble form of LMP is not. Furthermore, these data demonstrate that the detergent-soluble form of LMP is a functionally active form of the protein.

Before these studies, our model for LMP action and patch formation included a requirement for association with the detergent-insoluble cytoskeleton, or more specifically with vimentin intermediate filaments (9, 17). This model was supported by studies of the amino-terminally deleted form of LMP (D1LMP), in which a loss of functional activity in B-cell lines was associated with a loss of patch formation and loss of resistance to detergent extraction (17). The studies with Daudi cells presented here demonstrate that LMP can form patches and function without vimentin association. Thus, the functional defect of D1LMP is not likely due to its lack of cytoskeletal association. It is important to note that our assessment of LMP function is based on the measurement of a restricted set of cell activation parameters which LMP is known to affect. It is possible that there are other functions of LMP in B lymphocytes which are not yet defined and which absolutely require vimentin association.

LMP forms membrane patches possibly in association with B-lymphocyte membrane or membrane skeletal elements that normally function in receptor patch and cap formation. In cells which contain vimentin intermediate filaments, vimentin can form a tight association with the LMP patches, resulting in LMP and vimentin colocalization and resistance of LMP to detergent extraction. Thus, we now view the mutant D1LMP protein as primarily deficient in membrane patch formation. The ability of LMP to form

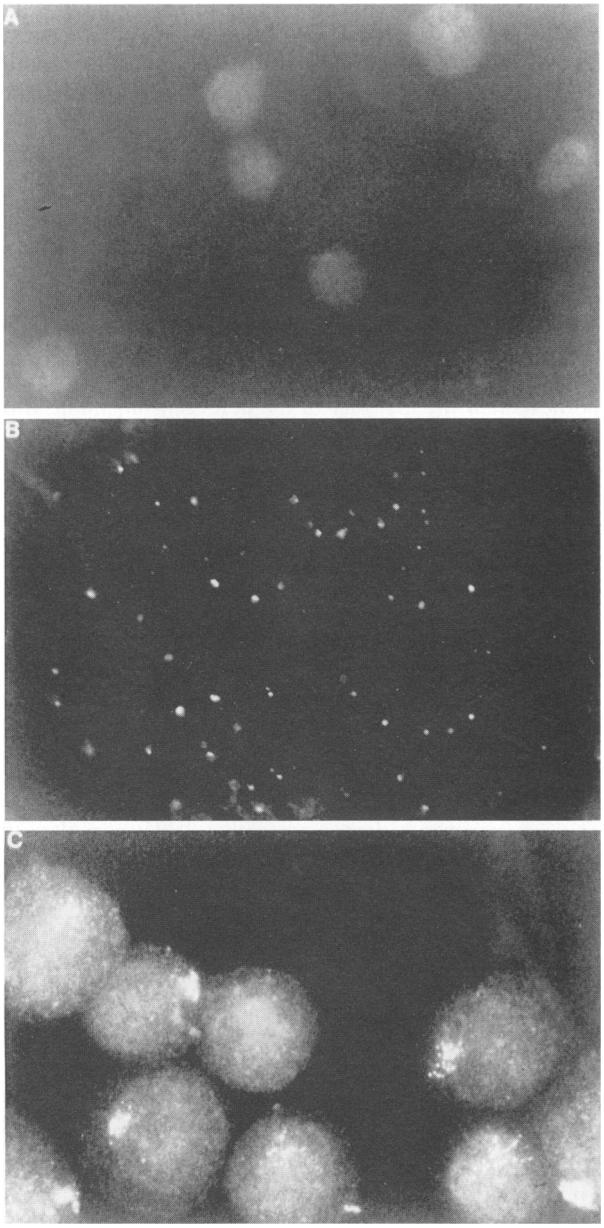

FIG. 4. LMP patch formation in Daudi cells. Daudi-gpt c.5 (A), Daudi-LMP c.3 (B), and Daudi-LMP c.17 (C) were fixed in methanol and stained for LMP by indirect immunofluorescence as previously described (10). Magnifications, ×400 (B) and ×1,000 (A and C).

patches in the membrane may be integral to its function in B lymphocytes. When LMP is introduced into cells which do not contain vimentin (Daudi cells), it still forms membrane patches; however, these patches never become resistant to detergent extraction as they do in vimentin-expressing cells. One possible role of the LMP-vimentin interaction may be to stabilize the LMP membrane patches.

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