

Epstein-Barr Virus Latent Infection Membrane Protein Increases Vimentin Expression in Human B-Cell Lines

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Latent Epstein-Barr virus (EBV) infection activates B-lymphocyte proliferation through mechanisms which are partially known. One approach to further delineate these mechanisms is to identify cellular genes whose expression is augmented in cells latently infected with EBV. Since EBV-negative Burkitt's lymphoma cells can be grown in continuous culture and EBV can establish growth-altering latent infection in these cells, some effects of EBV on B-lymphocyte gene expression can be studied by using this in vitro system. Pursuing this latter approach, we have used cDNA cloning and subtractive hybridization to identify a gene whose expression is increased after EBV infection. This gene encodes the cytoskeletal protein vimentin. Latent infection of established EBV-negative Burkitt's lymphoma cell lines with the transforming EBV strain, B95-8, resulted in dramatic increases in vimentin mRNA and protein levels, while infection with the nontransforming P3HR1 strain failed to do so. Vimentin induction was reproduced by the expression of the single EBV gene which encodes the latent infection membrane protein (LMP). An amino-terminal LMP deletion mutant did not induce vimentin. These results are of particular interest in light of the transforming potential of LMP, as demonstrated in rodent fibroblasts, and the interaction between vimentin and LMP observed in immunofluorescent colocalization and cell fractionation studies.

Epstein-Barr virus (EBV) causes infectious mononucleosis, a self-limited, polyclonal B-lymphocyte proliferation, and is associated etiologically with human neoplastic disorders (for a recent review, see reference 27). The putative oncogenic potential of the virus is likely related to its ability to establish latent infection and induce B-lymphocyte proliferation. Primary B lymphocytes infected in vitro are activated to proliferate indefinitely, resulting in immortalized lymphoblastoid cell lines (LCLs) (43). These cells maintain the viral genome as intranuclear episomes (37) and express a restricted set of EBV genes encoding several nuclear proteins (EBV nuclear antigens [EBNAs]) and at least one integral latent membrane protein (LMP) (for a recent review, see reference 10). The phenotypic changes after infection closely resemble those which occur as a consequence of mitogen stimulation (41). In both instances, surface antigens appear, including Ba (32), B6.4 (54), Blast-1 (52), B5 (17), BB-1 (59), CD23 (33, 51), Bac-1 (49), CD39 (AC-2 [46]), and 4F2 (26), which are absent or expressed at much lower levels in resting B cells. In addition, partially characterized autostimulatory growth factors have been identified in both EBV-infected and mitogen-activated B-lymphoblast supernatants (4, 6, 22, 23). These activation antigens and growth factors may play key roles in B-lymphocyte growth regulation, since specific monoclonal antibodies to these antigens and factors can alter B-cell proliferative responses (24).

Since EBV may transform B lymphocytes through latent gene product effects on B-lymphocyte regulatory macromolecules, we have begun to identify cellular genes whose expression is altered after infection. The initial phases of this study have been carried out in several laboratories using EBV-negative Burkitt's lymphoma (BL) cell lines infected in vitro to generate stable viral converted sublines expressing the characteristic array of latently expressed genes (7). Significantly, some of the activation antigens described

above, including CD23, CD39, and Bac-1, are not expressed in the parental EBV-negative BL lines but are induced after latent infection with the transforming EBV strain, B95-8 (7). Thus, although the parental cell lines are not dependent on EBV for continued proliferation in culture, latent viral infection is known to induce expression of many of the same proteins activated by EBV transformation. Moreover, EBV-negative BL cells transfected with EBV genes can be analyzed to determine whether individual viral genes effect specific phenotypic changes. Previous studies of this nature have demonstrated the induction of CD23 in a B-lymphoma line transfected with EBV nuclear antigen 2 (EBNA-2) (56) and induction of transferrin receptor and cell surface adhesion molecules, as well as CD23, in B cells transfected with EBV LMP (54).

B-lymphocyte genes overexpressed in EBV-infected cells were isolated by subtractive hybridization screening of a cDNA library that was prepared from the mRNA of an EBV-transformed LCL (IB4) (11). Polyadenylated RNA was prepared from EBV-negative BL cells (BL41 line) or from B95-8-infected BL41 (BL41/B95-8) cells in logarithmic growth by the guanidinium isothiocyanate-cesium chloride procedure (8) and oligodeoxythymidine cellulose column chromatography (2). Radiolabeled cDNA (2×10^8 cpm/ μ g of starting material) was synthesized from 5 μ g of BL41/B95-8 cell RNA, using avian myeloblastomatosis virus reverse transcriptase and [32 P]dCTP. After alkaline hydrolysis of the template RNA, the cDNA was hybridized with an excess of BL41 cell RNA for 24 h at 65°C in 0.5 M sodium phosphate buffer. Sequences corresponding to mRNA species present in both cell lines formed heteroduplexes of RNA-cDNA which were separated from single-stranded cDNA by isopycnic banding in 1.84 M CsSO₄ for 48 h (50). The single-stranded DNA fractions were pooled, dialyzed to remove residual CsSO₄, and subjected to a second cycle of subtraction and isopycnic banding. The remaining single-stranded cDNA, representing sequences expressed in BL41/B95-8

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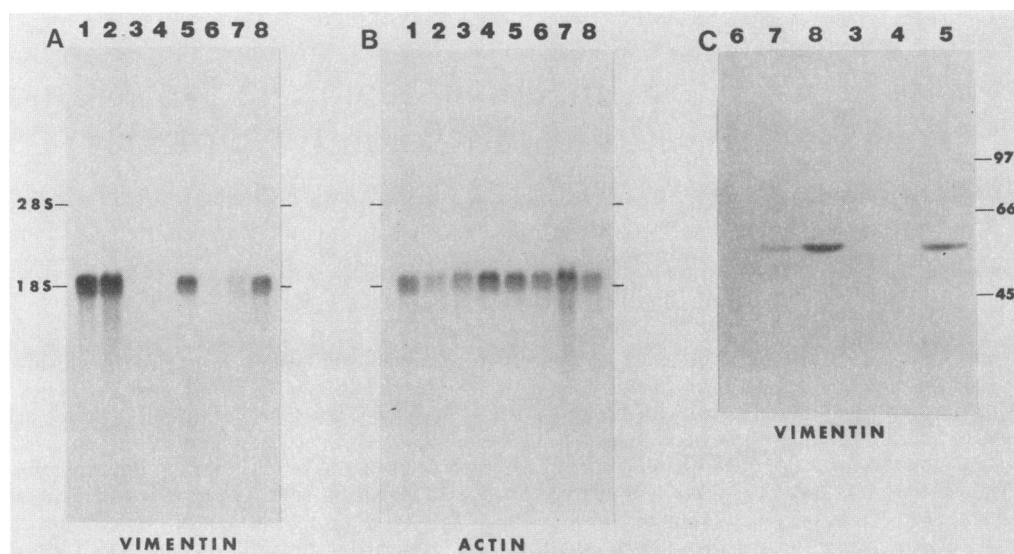


FIG. 1. Vimentin expression in BL cell lines and EBV-converted sublines. (A) Cytoplasmic RNA was prepared from cells in logarithmic growth, using the guanidinium isothiocyanate-cesium chloride method (8). RNA (12 μ g per lane) was size fractionated on a 1% formaldehyde-agarose gel, transferred to activated nylon membrane (Genescreen Plus, [Dupont, NEN Research Products]), and hybridized to a probe prepared from a human vimentin cDNA. (B) A duplicate blot was hybridized to a probe specific for gamma-actin as a control for the amount of RNA loaded per lane. After the filters were washed, they were exposed to preflashed film at -80°C , using a single intensifying screen for 48 h. The positions of the ribosomal markers (18S and 28S) are indicated at the sides of the radiographs by dashes. The cell lines used are as follows (A and B): lanes 1, IB4 (EBV latently infected lymphoblastoid cell line); lanes 2, unseparated tonsillar lymphocytes; lanes 3, BL30 (EBV-negative BL); lanes 4, BL30/P3HR1 (BL30 converted with nontransforming P3HR1 strain of EBV); lanes 5, BL30/B95-8 (BL30 converted with transforming B95-8 strain of EBV); lanes 6, BL41 (EBV-negative BL); lanes 7, BL41/P3HR1; lanes 8, BL41/B95-8. (C) Immunoblot for vimentin protein expression in BL cell lines and EBV convertants. Protein from 5×10^5 cells per lane was solubilized in a sample buffer consisting of 62 mM Tris hydrochloride (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, and 1 mM beta-mercaptoethanol. After being sonicated and boiled, proteins were electrophoresed on a sodium dodecyl sulfate polyacrylamide gel and transferred to nitrocellulose. The blot was reacted with a vimentin-specific monoclonal antibody, followed by rabbit anti-mouse immunoglobulin G and ^{125}I -protein A (Amersham Corp.). The numbers at the right indicate the molecular masses (in kilodaltons) of standard protein molecular mass markers. The cell lines represented in each lane are indicated at the top of the lanes, using the same number designations described above for the RNA blots.

cells but not in BL41 cells, constituted approximately 3% of the initial probe material. Although some of the probe loss through the subtraction procedures was nonspecific, the specificity of the probe was evident from subsequent hybridizations to 50,000 recombinant λ gt10 bacteriophage cDNA clones (47). After initial isolation by hybridization with the subtractive probe, clones of EBV-induced genes were confirmed by probing duplicate nitrocellulose filters with the subtracted ^{32}P -labeled BL41/B95-8 cDNA and with ^{32}P -labeled BL41 cDNA, respectively. A single phage clone was isolated which consistently hybridized with BL41/B95-8 cDNA but showed no reactivity with BL41 cDNA. The insert was subcloned into pBluescript and sequenced by the dideoxy method (48). The sequence was compared with the GenBank data base and was found to be nearly identical to the published sequence of a human vimentin cDNA, with minor differences in the 3' untranslated region (16).

To ascertain whether elevated expression of vimentin mRNA was a consistent feature of latent EBV infection, the vimentin cDNA was labeled with ^{32}P by the random hexanucleotide primer method (14) and hybridized to Northern (RNA) blots onto which RNA from a variety of EBV-positive and -negative cell lines had been transferred. The probe identified a 2.0-kilobase mRNA, which was present at significantly higher levels in BL30 converted with transforming strain B95-8 of EBV (BL30/B95-8) (Fig. 1A, lane 5) and BL41/B95-8 (lane 8) cells than in the EBV-negative parental lines BL30 (lane 3) and BL41 (lane 6). A high level of

vimentin expression was also seen in the EBV-transformed LCL, IB4 (lane 1), as well as in unfractionated tonsillar lymphocytes. It should be noted that the latter represents RNA derived from a heterogeneous population of cells, including resting and activated B lymphocytes, T lymphocytes, dendritic cells, and stromal cells, any or all of which may be expressing high levels of vimentin. Roughly equivalent amounts of mRNA were loaded in each lane as shown by hybridization of a parallel blot with a gamma-actin-specific probe (Fig. 1B). Comparable induction of the 55-kilodalton (kDa) vimentin protein was observed in the B95-8-infected cell lines, as determined by Western blot (immunoblot) using a vimentin-specific monoclonal antibody (Fig. 1C).

A variant EBV strain, P3HR1, with EBNA-2 and a portion of the EBNA leader protein (EBNA-LP [47]) genes deleted, lacks the ability to transform B lymphocytes (38, 44). Although the P3HR1 deletion does not involve the open reading frames of LMP or any of the other known latent infection genes, since these map to distant loci in the EBV genome, P3HR1-infected BL41 and BL30 cells have previously been shown also to lack detectable LMP expression (40). The failure of the P3HR1 convertants to express LMP could be a result of the absence of EBNA-2 or the lack of a complete and functional EBNA-LP or the result of a defect in the P3HR1 LMP gene. This viral strain failed to induce vimentin expression in BL30 cells and induced it only weakly in BL41 cells (Fig. 1). The differential response to

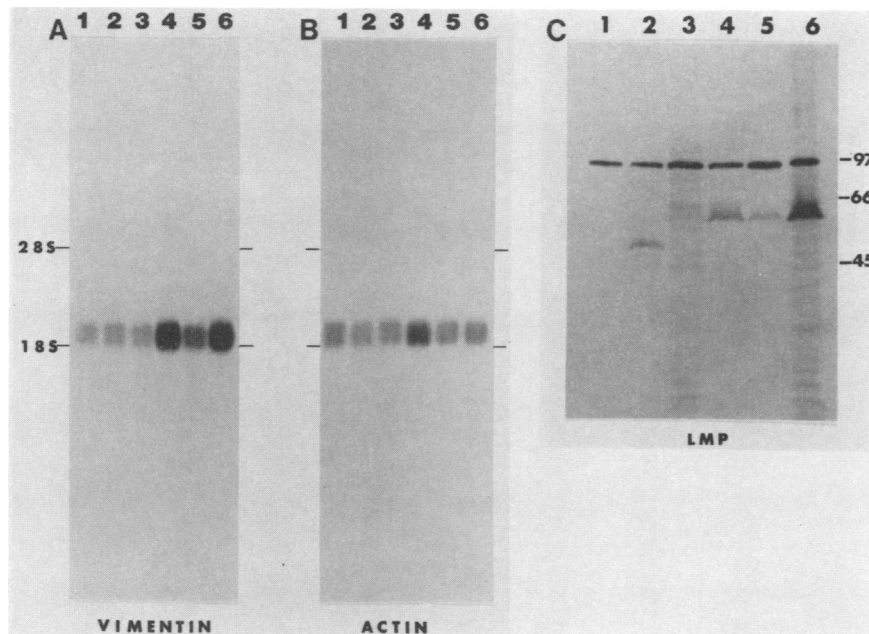


FIG. 2. Vimentin expression in Louckes cells and Louckes LMP transfectants (52, 54). Cytoplasmic RNA (15 μ g per lane) was prepared from Louckes cell-derived transfectant clones maintained under guanine phosphoribosyltransferase selection in the presence of 2.5 μ g of mycophenolic acid per ml. The cell lines used are as follows (A and B): lanes 1, Louckes gpt1 (control transfected with pSV2 gpt vector); lanes 2, Louckes XL1 (D1LMP transfectant); lanes 3 through 6, Louckes LM1, LM2, LM3, and LM6, respectively (LMP transfectants). Duplicate blots were prepared and hybridized to probes specific for vimentin (A) and gamma-actin (B). (C) Levels of LMP protein expression were analyzed by immunoblot, using the LMP-specific monoclonal antibody S12. The contents of each lane are indicated by the numbers at the top of the lanes, using the same designations described above for the RNA blots. The numbers to the right indicate the molecular masses (in kilodaltons) of standard protein markers. LMP is the 60-kDa protein, while D1LMP is the 49-kDa protein. The 95-kDa protein is a cross-reactive cellular protein present in all cell lines tested.

P3HR1 infection compared with that of B95-8 infection may indicate that high-level vimentin induction is a specific effect of the transforming phenotype and not merely the result of nonspecific alterations consequent to viral infection. The minor enhancement in vimentin expression seen in P3HR1-infected BL41 (BL41/P3HR1) cells may relate to the weak activational properties of this virus, as evidenced, for example, in the intermediate level of induction of the activation antigen, CD39, in most P3HR1 BL convertants (7).

To determine whether individual viral genes might directly affect vimentin expression, we analyzed vimentin mRNA levels in a previously described EBV-negative BL line (Louckes), transfected with genes encoding EBNA-2, EBNA-LP, or LMP (57, 58). The EBNA-2 or EBNA-LP genes were expressed under control of the Moloney murine leukemia virus promoter (58) in the EBV-negative BL cell line. The human metallothionein type 2 promoter was used for LMP gene expression (56, 57). The EBV genes were also linked to a selectable marker gene encoding either neomycin phosphotransferase or guanine phosphoribosyltransferase, and transfected cells were maintained under positive selection. These constructs result in EBNA-2, EBNA-LP, or LMP expression levels in Louckes cells similar to those ordinarily found in EBV-transformed lymphocytes. Northern blot analysis demonstrated a marked elevation in steady-state vimentin mRNA levels only in Louckes cells expressing LMP (Fig. 2; also data not shown). Similar effects were observed with LMP expression in BL41 cells (Fig. 3). Significantly, the magnitude of vimentin induction correlated with the level of LMP protein expression in both Louckes and BL41 cells. This is most evident when comparing the BL41 LM11 clone with BL41 LM2 and Louckes LM6 with

Louckes LM3. The Louckes LM1 line expressed a small amount of an aberrantly sized LMP and showed no vimentin induction.

A potential initiation codon in the fourth LMP transmembrane domain is used to initiate translation of a form of LMP that is shorter at the amino terminal end in cells permissive for EBV replication (29). This natural deletion derivative (D1LMP) has been of interest because it is inserted in cell membranes but lacks rodent cell transforming and lymphocyte activation activities (56). Most significantly, vimentin mRNA was not induced in cells transfected with D1LMP. Expression of D1LMP protein in these cells, as determined by Western blot (Fig. 3C, lane 2), equaled or exceeded the level of LMP expression seen in the Louckes LM3 line. The latter, however, clearly exhibited elevated vimentin mRNA levels relative to that of the vector control.

These experiments demonstrate that latent infection by EBV in B lymphocytes is associated with a dramatic enhancement of vimentin expression evident at both the protein and mRNA levels. This induction is dependent upon expression of the EBV LMP and can be fully reconstituted in EBV-negative BL cells which express no EBV gene other than that encoding LMP.

Primary sequence analysis, protease digestion, and monoclonal antibody reactivity studies indicate that LMP is an integral membrane protein with six putative membrane-spanning hydrophobic domains separated by five short reverse turns, a short cytoplasmic amino terminus, and a long cytoplasmic, negatively charged carboxy terminus (15, 36). LMP appears to play a pivotal role in EBV-mediated transformation. Transfection of LMP into immortalized rodent fibroblasts results in anchorage-independent growth and

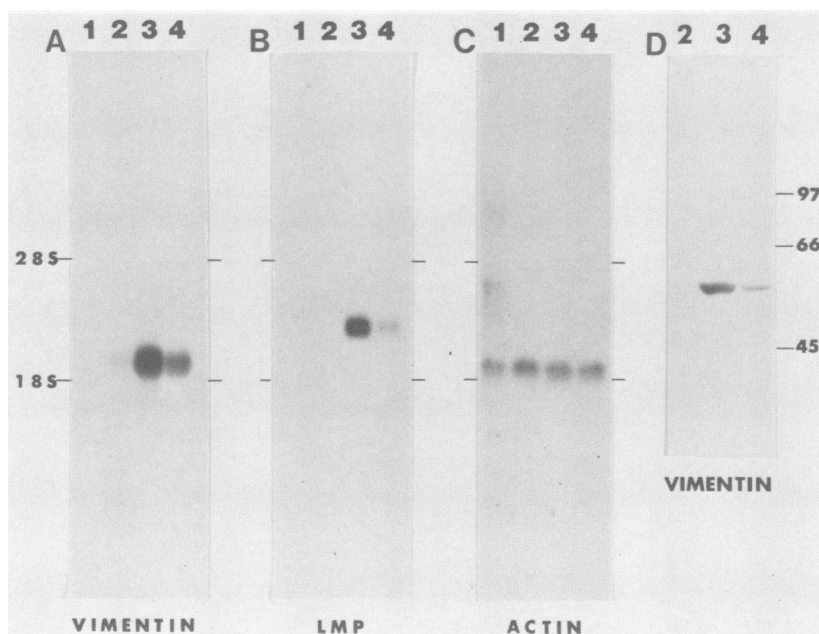


FIG. 3. Vimentin expression in BL41 cells and BL41 LMP transfectants. Cytoplasmic RNA (15 μ g per lane) was prepared from BL41 cells (lanes 1) and a series of BL41-derived transfectant clones: BL41 gpt2 (control transfected with pSV2 gpt vector [lanes 2]); BL41 LM2 (LMP transfectant [lanes 3]); and BL41 LM11 (LMP transfectant [lanes 4]). Transfectant cell lines were cultured under continuous guanine phosphoribosyltransferase selection in the presence of 2.5 μ g of mycophenolic acid per ml. Three identical blots were prepared and hybridized to probes specific for vimentin (A), LMP (B), and gamma-actin (C). The positions of the rRNA bands (18S and 28S) are indicated by dashes at the sides of the autoradiographs. (D) Vimentin protein expression was analyzed in the BL41 transfectants by immunoblot, using a vimentin-specific monoclonal antibody. Proteins were prepared as described above from each cell line at the time of harvesting for RNA extraction. The contents of each lane are indicated by the numbers at the top of the lanes, as designated above for the RNA blots. The numbers to the right indicate the molecular masses (in kilodaltons) of standard protein markers.

acquisition of tumorigenic properties in nude mice (3, 55). LMP expression in EBV-negative BL cells results in the induction of a number of surface markers which characterize EBV-transformed lymphocytes, including the transferrin receptor and the adhesion molecules LFA-1, ICAM-1, and LFA-3 (57). Moreover, these cells demonstrate higher levels of intracellular calcium than control cells transfected with an expression vector only (57). None of these phenotypic changes occur in cells expressing the amino-terminally truncated D1LMP, which is normally expressed only in the virus replication cycle.

Previous studies have demonstrated a close physical interaction between LMP and the lymphocyte vimentin cytoskeletal network (35). Double immunofluorescence labeling revealed that vimentin, which is normally evenly distributed in radial cytoplasmic fibers in most resting B cells (13, 35, 42), is redistributed in EBV-transformed and latently infected cells, in part forming aggregates beneath LMP plasma membrane caps and patches (35). This colocalization of vimentin with LMP patches requires only LMP and not other EBV genes, since it is observed in cells transfected with the LMP gene alone (57). Not only does LMP cause vimentin to localize near it, but vimentin can alter LMP localization. Treatment with colcemid results in the collapse of the vimentin network into perinuclear rings and relocation of LMP to these rings (35). Moreover, a significant proportion of the LMP in LCLs and EBV-transformed BL cell lines is resistant to repeated extractions with nonionic detergents, functionally defining this fraction as cytoskeleton associated (35).

Little is known regarding the transcriptional regulation of vimentin or its function. In fibroblasts, vimentin mRNA

levels increase dramatically within 4 to 6 h after exposure to platelet-derived growth factor or serum stimulation (28). This response is unchanged in the presence of cycloheximide (45), indicating that it is not dependent upon new protein synthesis. Thus, with respect to the characteristics of its response to mitogens, vimentin resembles other known cell cycle-dependent genes, such as *c-myc* (31) and *KC* (9). Immunohistochemical analysis of human lymphoid tissues and peripheral blood indicates that vimentin filaments are present in all mature T cells, forming evenly distributed radial networks in the majority of these (12, 13, 42). B cells of lymph node mantle zones and peripheral blood also make vimentin (39). It is undetectable, however, in germinal center B cells (39), although these are presumed to be proliferating. Postfollicular cytoplasmic immunoglobulin-positive immunoblasts and plasma cells express vimentin at low levels in a perinuclear distribution (13, 39). In vitro activation of unfractionated peripheral blood mononuclear cells with phytohemagglutinin results in rapid vimentin mRNA induction (30). This induction appears to be T cell specific. Interestingly, treatment of purified T cells depleted of accessory cells with phytohemagglutinin results in phenotypic changes indicative of blast transformation but is insufficient to drive cells to enter S phase and does not induce vimentin (30). It is not known whether vimentin expression is altered in mitogen- or antigen-activated B cells, although it is weakly induced in BL cells treated with tetradecanoylphorbol acetate (C. Alfieri, unpublished observation).

There are several ways in which the observed effects of LMP on vimentin expression could be explained. LMP could induce vimentin directly through processes mediated via its cytoskeletal association. A specific testable hypothesis is

that LMP induces vimentin expression by localizing to the plasma membrane a vimentin fraction which otherwise acts at another site to repress vimentin expression. Alternatively, LMP could induce vimentin indirectly, along with B-cell activation antigens, through pleiotropic or sequential cascade effects of B-cell activation. Lastly, LMP expression could exert toxic effects on BL cells which vimentin expression could protect against, resulting in selective outgrowth of higher vimentin expressing clones. If this were the case, LMP-expressing clones should arise at a lower frequency than vector control clones derived in parallel experiments, and control clones should be heterogenous in vimentin expression. In fact, the frequency with which LMP-expressing clones were derived during selection after transfection did not differ from the frequency of vector control clones, and none of the four control clones which we examined differed in vimentin expression from characteristically low levels. Therefore, this last hypothesis is unlikely.

It has been suggested that vimentin may play a role in the transduction of signals from the plasma membrane to the nucleus (53). *In vitro* studies using nuclear and plasma membranes purified from avian erythrocytes have shown that vimentin can interact with the plasma membrane protein ankyrin via its amino terminus and that vimentin can interact with the nuclear matrix protein lamin B via its carboxy terminus (18, 19). Human vimentin, in similar studies, has also been shown to bind erythrocyte ankyrin *in vitro* via its amino terminus (20, 21). Activation of B cells by cross-linking of surface immunoglobulin profoundly alters cytoskeletal architecture. Vimentin filaments redistribute into submembranous aggregates associated with the surface immunoglobulin cap, reminiscent of the appearance of EBV-transformed cells (12). Under such conditions, surface immunoglobulin becomes associated with the cytoskeleton, as demonstrated by resistance to detergent extraction (5). Significantly, among various antiimmunoglobulin monoclonal antibodies, the ability to cap surface immunoglobulin is directly correlated with efficiency in driving cells into mitosis (25), and induction of surface immunoglobulin association with the cytoskeleton (1). Recently, LMP has been shown to exert B-cell activation effects in the absence of vimentin association (34). Thus, vimentin is not absolutely required for many LMP activities in B lymphocytes. However, there may be important, as yet uncharacterized, LMP functions which do require an LMP-vimentin association. Alternatively, vimentin may be important in regulating LMP of normal B lymphocytes. Such putative functions may not be apparent or relevant in LMP-expressing BL cell lines. In this regard, it is noteworthy that D1LMP does not demonstrate any interaction with the vimentin cytoskeleton and does not transform rodent fibroblasts or induce B-lymphocyte activation (55, 56).

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