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Transformation by the Oncogenic Latent Membrane Protein Correlates with Its Rapid Turnover, Membrane Localization, and Cytoskeletal Association

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The latent membrane protein (LMP) of Epstein-Barr virus (EBV) has a short half-life (V. R. Baichwal and B. Sugden, J. Virol. 61:866–875, 1987; K. P. Mann and D. Thorley-Lawson, J. Virol. 61:2100–2108, 1987), is localized in patches in the membrane (D. Liebowitz, D. Wang, and E. Kieff, J. Virol. 58:233–237, 1986), and associates with the cytoskeleton in EBV-immortalized B lymphocytes (D. Liebowitz, R. Kopan, E. Fuchs, J. Sample, and E. Kieff, Mol. Cell. Biol. 7:2299–2308, 1987; K. P. Mann and D. Thorley-Lawson, J. Virol. 61:2100–2108, 1987). Deletion mutants of LMP that are either positive or negative in the induction both of anchorage-independent growth of BALB/c 3T3 cells (V. R. Baichwal and B. Sugden, Oncogene 4:67–74, 1989) and of cytotoxicity in a variety of cells (W. Hammerschmidt, B. Sugden, and V. R. Baichwal, J. Virol. 63:2469–2475, 1989) have been studied to identify the biochemical properties of this protein that correlate with its effects on cell growth. Mutant LMP proteins that are metabolically stable, do not associate with the cytoskeleton, and exhibit a diffuse plasma membrane localization also do not induce anchorage-independent growth in rodent cells or cytotoxicity in B lymphoblastoid cells. In contrast, a mutant of LMP that is functionally identical to the wild-type protein has a half-life, membrane localization, and cytoskeletal association similar or identical to those of LMP. These results are consistent with the hypothesis that LMP's rapid turnover, association with the cytoskeleton, and patching in the membrane are required for it to affect cell growth.

Epstein-Barr virus (EBV) is a human herpesvirus which infects B lymphocytes and epithelial cells in vivo and immortalizes B lymphocytes in vitro. This virus is associated with three human tumors, African Burkitt's lymphoma, nasopharyngeal carcinoma, and B-cell lymphomas, in immunocompromised hosts (22). Infection of resting human B lymphocytes in vitro with EBV results in their being immortalized; these infected cells proliferate indefinitely (22). EBV is maintained in a latent state in the immortalized cell; of the latently expressed viral genes, at least two are implicated in the process of immortalization. Strong evidence indicates that the LMP (latent membrane protein; previously referred to as BNLF-1 [1]) oncogene of EBV is likely to contribute to the process of immortalization of the infected cell. Introduction of the LMP gene driven by heterologous promoters into rodent fibroblasts confers upon these cells the ability to grow in soft agarose; the resulting transformed cells are tumorigenic in nude mice (2, 27). The transforming domain of LMP is cytotoxic when expressed at a high level in a variety of cell lines (10). In addition, the LMP gene can alter the phenotype of EBV-negative lymphoblastoid cell lines (29) and can also affect the growth properties of human epithelial cell lines (5, 6). It is probable that the biochemical activity of LMP that renders it oncogenic in rodent cells will contribute also to immortalization of B lymphocytes by EBV. An understanding of the mechanism by which LMP can alter the growth properties of rodent cells should therefore provide information that will facilitate the study of LMP signaling pathways in human B lymphocytes which are experimentally less tractable.

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The biochemical activity associated with the transforming function of LMP is unknown. As predicted from its DNA sequence (4, 7), LMP is a 62-kDa integral membrane protein possessing a predicted tertiary structure similar to that of the rhodopsin family of cell surface receptors and recently identified ion channel proteins (4, 11, 20). LMP is predicted to have six membrane-spanning domains, and its carboxy terminus has been shown to be on the cytoplasmic side of the membrane (11, 17, 20). The LMP protein is present in punctate patches in the membrane (17, 20), is phosphorylated on the carboxy terminus at serine and threonine residues (1, 21), and is associated with the detergent-insoluble cytoskeleton (16, 21). In addition, LMP is a short-lived protein with a half-life of 2 to 5 h both in rodent cell lines rendered anchorage independent by the LMP gene (2, 21a) and in EBV-positive lymphoblastoid cell lines (1, 21). The relationship of these properties to the transforming activity of LMP and the biochemical basis for this transforming activity are not understood.

We have undertaken a series of experiments designed to explore the relationship between the transforming function of the LMP protein and its rapid turnover, membrane localization, and association with the cytoskeleton. The results of these experiments demonstrate that the turnover, cytoskeletal association, and membrane localization of LMP correlate with its transforming function. LMP apparently must exhibit these three biochemical properties in order to function (i.e., transform cells).

MATERIALS AND METHODS

Cells. HEp-2 is an adherent human epithelial cell line and was obtained from the American Type Culture Collection.

BALB/c 3T3 is an adherent murine fibroblast cell line obtained from the American Type Culture Collection. 721 is a B cell line transformed in vitro by infection with EBV (12). Cells were grown in 5% CO_2 atmosphere at 37°C in Dulbecco's medium supplemented with 10% bovine calf serum (Hyclone Laboratories), 200 U of penicillin per ml, and 200 μg of streptomycin per ml; Dulbecco's medium was substituted with RPMI 1640 for growth of 721 cells. The LMP-transformed BALB/c 3T3 cell line (BALB cl. 5) was derived by Baichwal and Sugden by cotransfection of BALB/c 3T3 cells with pSV_2-LMP plus pSV_2-Neo (2). Briefly, transfected cells were selected with 500 μg of G418 per ml for 3 weeks, and G418-resistant colonies were pooled and plated in agarose as described previously (2). After 3 weeks in agarose, anchorage-independent colonies were picked and propagated on tissue culture plastic. To obtain BALB/c 3T3 cell lines expressing both LMP and mutant LMP proteins, BALB cl. 5 was transfected with expression vectors containing both the mutant LMP gene and the gene for hygromycin resistance (pCMV-Δ25-132, pCMV-Δ27-212, and pCMV-NΔ43). Transfected cells were selected with 400 μg of hygromycin (Calbiochem) per ml for 3 weeks, and colonies were picked and propagated. To obtain BALB/c 3T3 cell lines expressing the CΔ55 mutant, cells were cotransfected with pSV_2-CΔ55 and CG214X-SVHy (described below) and selected with 400 μg of hygromycin per ml for 3 weeks; colonies were picked and expanded into stable cell lines.

Plasmids. The LMP and mutant expression vectors pSV_2-LMP, pSV_2-NΔ43, and pSV_2-Δ27-212 have been described previously (2, 3); pSV_2-BNLF-1 has been renamed pSV_2-LMP. pSV_2-LMP encodes the LMP gene expressed from the simian virus 40 (SV40) enhancer and early promoter. The mutants described above were derived from pSV_2-LMP. The LMP and mutant expression vectors pCMV-LMP, pCMV-NΔ43, pCMV-Δ25-132, pCMV-Δ27-212, and pSV_2-Δ25-132 have been described previously (10). The cytomegalovirus (CMV) expression vectors contain the immediate-early promoter and enhancer from human CMV (24). pCMV-BNLF-1 has been renamed pCMV-LMP. pSV_2-CΔ55 was derived from pSV_2-LMP and has an *Xba*I linker with termination codons in all three reading frames inserted at the *Bst*EII site in the LMP gene. pCMV-CΔ55 was derived from pCMV-LMP and has an *Xba*I linker with termination codons in all three reading frames inserted at the *Bst*EII site in the LMP gene. The CΔ55 mutant lacks 55 amino acids from the carboxy terminus of LMP. pCMV-NΔ25 was derived from pCMV-NΔ9 (10) by deletion of sequences between the *Xba*I site present in sequences 5′ to the LMP gene and the *Stu*I site within the LMP gene, followed by insertion of a *Cla*I linker d(CCCATCGATGGG) at the position of the *Stu*I site to provide an ATG in frame with the LMP sequence. The amino acid residue following the initiating methionine provided by the *Cla*I linker is glycine, which corresponds to the 26th residue in LMP. CG214X-SVHy is a spleen necrosis virus-based vector that encodes the gene for hygromycin resistance expressed from the SV40 early promoter (7a).

Transfections. HEp-2 cells were transfected by the use of DEAE-dextran as follows. Cells (50% confluent 100-mm dish; ~2 × 10^6 cells) were incubated in 1 ml of Dulbecco's medium containing DEAE-dextran (500 μg/ml) and plasmid DNA (2 to 4 μg/ml) for 45 min at 37°C in a humidified atmosphere of 5% CO_2. The DEAE-dextran solution was removed, and cells were washed two times in phosphate-buffered saline (PBS) and incubated in Dulbecco's medium containing 2% bovine calf serum (Hyclone) and 100 μM chloroquine (Sigma) for 3 h at 37°C. After removal of this solution, cells were incubated in Dulbecco's medium containing 10% bovine calf serum until time of harvest (48 h after transfection). BALB/c 3T3 cells were transfected by electroporation as previously described (2).

Anchorage-independent growth assay. Transformation assays were done as previously described by Baichwal and Sugden (2) and modified by Hammerschmidt et al. (10).

Cytotoxicity assay. LMP-mediated cytotoxicity was measured in HH514 cells (EBV-positive lymphoblastoid cell line) as previously described (10).

Immunofluorescence. BALB/c 3T3 cells transfected with LMP expression vectors were washed in PBS and fixed in acetone-methanol (1:1) at −20°C for 20 min. Fixed cells were blocked with PBS–1% calf serum, incubated with a 1:20 dilution of an affinity-purified anti-LMP antiserum (1), a 1:500 dilution of antitubulin rat monoclonal antibody (YL1/2; Accurate Chemical and Scientific Corp.), or a 1:20 dilution of antivimentin (ICN) at 37°C for 45 min, washed in PBS–1% calf serum, and incubated with a 1:40 dilution of fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin (Sigma), a 1:40 dilution of Texas red-conjugated goat anti-rat immunoglobulin (Kirkegaard & Perry Laboratories, Inc.), or a 1:40 dilution of fluorescein isothiocyanate-conjugated rabbit anti-goat immunoglobulin (Sigma) for 45 min at 37°C. After being washed in PBS–1% calf serum and mounted for viewing, cells were visualized by using a Zeiss microscope and photographed at a magnification of ×1,000.

Pulse-chase analysis. Cells grown to 50% confluence on 100-mm plates were incubated in 1 ml of methionine-free RPMI–10% dialyzed fetal calf serum for 30 min at 37°C and then incubated in the same medium containing Tran[^{35}S]-label(methionine-cysteine) (250 μCi/ml; 1,150 Ci/mmol; ICN) for 1 h. Plates were then washed three times in PBS and incubated in Dulbecco's medium–10% bovine calf serum for the duration of the chase (the concentrations of methionine and cysteine in Dulbecco's medium–10% calf serum are at least 600- and 2,400-fold, respectively, higher than that contributed by ^{35}S label during the pulse). At the time of harvest, cells were washed three times in PBS, detached from the plate by trypsinization, and collected by centrifugation.

Immunoprecipitation. ^{35}S-labeled cell pellets were solubilized in 5× RIPA buffer (1× RIPA is 0.15 M NaCl, 1% [vol/vol] Triton X-100, 0.5% [wt/vol] sodium deoxycholate, 0.5% [wt/vol] sodium dodecyl sulfate [SDS], 1% [vol/vol] Trasylol [FBA Pharmaceuticals], 1× protease inhibitor mix [20 μM *N*-α-*p*-tolyl-L-lysine chloromethyl ketone, 5 mM 6-amino caproic acid, 2 mM EDTA, 100 μM benzamidine, 5 μg of leupeptin per ml, 7 μg of pepstatin per ml, and 1,000 U of Trasylol] [23], and 0.01 M Tris, pH 7.2) by sonication and diluted from 5× RIPA to 1× RIPA with sterile distilled H_2O. Lysates were boiled for 10 min prior to immunoprecipitation. Lysates were immunoprecipitated by a method modified from Sugden and Metzenberg (25) and Baichwal and Sugden (1). Generally, one half of a cell lysate (equivalent to ~10^6 cells) in a volume of 0.5 ml was immunoprecipitated. Lysates were precleared by incubation with 0.35 ml of Bio-Rad Immunobead reagent (goat anti-rabbit immunoglobulin, heavy and light chain specific, reconstituted in 50 ml of 1× RIPA buffer) in a total volume of 0.75 ml of 1× RIPA buffer for 20 min at 0°C, followed by centrifugation in a microfuge (Fisher) for 5 min to remove the beads. The supernatant was removed, respun, and incubated with 130 μl of affinity-purified anti-LMP antiserum for 30 min at 0°C. LMP was precipitated by incubation with 0.15 ml of Immu-

nobead reagent for 30 min at 0°C. Beads were washed four times in 1× RIPA buffer and boiled in SDS sample buffer (13) for 10 min. Samples were separated on 10% acrylamide gels, soaked in Enhance (New England Nuclear), dried, and exposed to Kodak XAR-5 film at −70°C. The half-life of LMP was determined by laser densitometric scanning of fluorographs from plots of log area under the curve (arbitrary units) versus hours of chase which were linear, with correlation coefficients of ≥0.98. The amount of LMP lost in the preclearing step of this procedure is ~5% as determined by Western immunoblot analysis (data not shown), and the majority of the Triton-insoluble fraction of LMP has been shown to be soluble in 1× RIPA buffer (1).

TCA precipitation of ^{35}S-labeled cell lysates. ^{35}S-labeled cell extracts (~10^5 cpm taken from the 0-h time point of chase; 0.1% of total lysate) were incubated in 12% trichloroacetic acid (TCA) for 30 min at 25°C, filtered over Whatman GF/C glass fiber filters, and counted in liquid scintillation cocktail. The half-life of total protein was determined from plots of log TCA-precipitable counts per minute versus hours of chase which were linear, with correlation coefficients of ≥0.98.

SDS-PAGE and Western analysis. LMP and mutant LMP proteins were resolved by electrophoresis through 10% polyacrylamide gels (PAGE; 13). Prestained molecular size markers were from Bethesda Research Laboratories and consisted of myosin heavy chain (200,000 kDa), phosphorylase *b* (97,400 kDa), bovine serum albumin (68,000 kDa), ovalbumin (43,000 kDa), and α-chymotrypsinogen (27,700 kDa). Proteins separated by SDS-PAGE were transferred to nitrocellulose as described previously (18). Nitrocellulose filters were blocked in PBS–0.05% Tween 20–1% Carnation instant nonfat milk and stained with a 1:100 dilution of the affinity-purified anti-LMP antiserum in PBS–0.05% Tween 20–1% milk for 1 h at 37°C. After washing to remove the primary antibody, blots were incubated in a 1:500 dilution of alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (Sigma) in PBS–0.05% Tween 20–1% milk for 1 h at 37°C. Blots were then washed with PBS–0.05% Tween 20–1% milk and developed by using a combination of nitroblue tetrazolium (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma) as described previously (14).

Triton X-100 fractionation. HEp-2 cells transiently transfected with LMP expression vectors, or BALB/c 3T3 cells stably transfected with LMP expression vectors, were assayed for LMP solubility in 1% Triton X-100 as follows. Cells (~2×10^6 cells on 100-mm plates; 50 to 75% confluent) were washed three times in PBS and incubated at 25°C in 0.5 ml of a buffer containing 1% Triton X-100 (Sigma), 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.2), 50 mM KCl, 2.5 mM $MgCl_2$, 0.3 M sucrose, 1% Trasylol, and 1× protease inhibitor mix for 3 min with rocking every 30 s, and the solubilized material was removed and kept on ice (Triton wash 1). This wash was repeated two more times for 1 min each time, with rocking every 30 s (Triton washes 2 and 3). The Triton-insoluble material left on the plate was removed by solubilization in 0.5 ml of 1× RIPA buffer (Triton insoluble). The total LMP on an unfractionated plate of cells was solubilized in 0.5 ml of 1× RIPA (unfractionated) and used as a measure of the total LMP in the sample. These extracts were sonicated on ice, diluted into SDS sample buffer, boiled for 10 min, and loaded onto 10% polyacrylamide gels. Gels were transferred to nitrocellulose and stained with the anti-LMP antiserum. To ensure linearity of the assay, fivefold serial dilutions of unfractionated lysate from each fractionation were loaded on each gel, and only those signals falling within the linear portion of this standard curve were considered to be valid. In control experiments, the Triton washes (prior to sonication) were centrifuged for 10 min in a microfuge to remove whole cells that may have become detached during the washes. There was no difference in levels of LMP protein detected by Western analysis upon comparison of Triton washes that were centrifuged with those that were not (there is no visible pelleted material after such treatment) (data not shown). Therefore, the Triton-soluble LMP samples do not have contaminating LMP derived from whole cells. The LMP in the Triton-insoluble material was visualized by immunofluorescence as follows. Cells were washed in the 1% Triton X-100 buffer to remove Triton-soluble LMP (as described above), washed three times in the same buffer lacking Triton X-100, and then fixed in acetone-methanol (1:1) for 20 min at −20°C. Fixed cells were stained for LMP as described above.

Nocodazole treatment of intact cells. Intact cells (50% confluent) were incubated in Dulbecco's medium–10% bovine calf serum containing 50 ng of nocodazole per ml for 30 to 60 min at 37°C. Cells were either fixed for immunofluorescence as described above (data not shown) or fractionated in 1% Triton X-100 as described above. Immunofluorescence staining of nocodazole-treated cells with antibodies to tubulin revealed that a 30-min treatment was sufficient to depolymerize microtubules in the cell (data not shown). Untreated cells exhibited a characteristic organized filamentous tubulin staining pattern, while nocodazole-treated cells had a nonfilamentous, disorganized, cytoplasmic tubulin staining pattern characteristic of depolymerized tubulin (8).

RESULTS

Deletion mutants of LMP. The relationship between the transforming function of LMP and its turnover, membrane localization, and association with the cytoskeleton was investigated by using deletion mutants of LMP (Fig. 1). These mutants score as being either positive or negative in induction of anchorage-independent growth of BALB/c 3T3 cells (3, 10) and of cytotoxicity in a variety of cell types, including B lymphoblastoid cells (10). The LMP and mutant LMP genes are cloned into each of two eukaryotic expression vectors and are expressed from either the SV40 early promoter and enhancer to achieve a moderate level of expression (2) or the human CMV immediate-early promoter and enhancer to achieve a high level of expression (10). The biological activity of the deletion mutants as assayed by induction both of anchorage-independent growth in BALB/c 3T3 cells and of cytotoxicity in lymphoblastoid cells is indicated in Fig. 1 as described in the legend.

The carboxy-terminal deletion mutants (CΔ199, CΔ174, and CΔ155) described previously (3) are difficult to detect when expressed in both transient and stable assays, since they lack most epitopes recognized by the anti-LMP antiserum that was raised against the carboxy terminus of LMP (1). For this reason, a new mutant was constructed that lacks 55 amino acids from the carboxy terminus, leaving a sufficient number of epitopes intact such that recognition by the antiserum is not affected. This mutant was tested for induction of anchorage-independent growth in BALB/c 3T3 cells and cytotoxicity in an EBV-positive lymphoblastoid cell (HH514) (10). Transfection of pSV_2-CΔ55 into BALB/c 3T3 cells resulted in the induction of 85 anchorage-independent colonies per 2×10^6 cells plated in agarose after 3 weeks of growth, compared with 75 anchorage-independent colonies

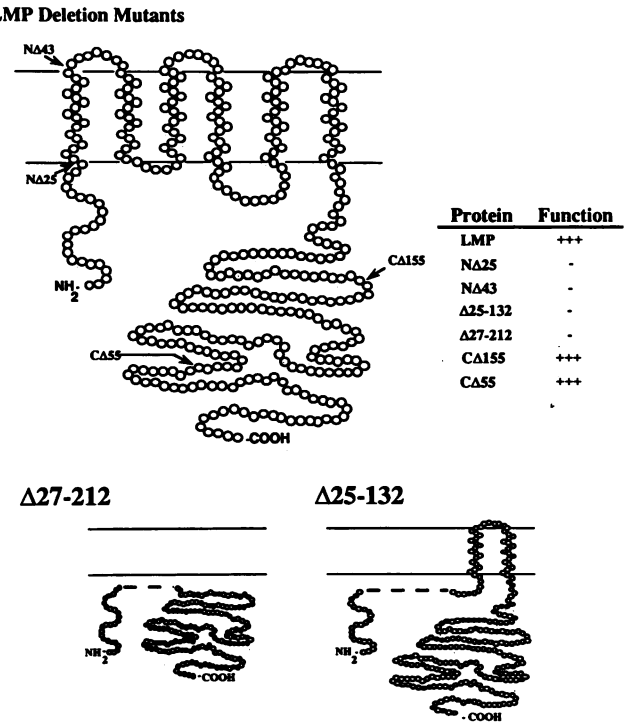


FIG. 1. LMP deletion mutants. A schematic representation of the predicted LMP structure is shown, with amino and carboxy termini depicted on the cytoplasmic side of the plasma membrane. Locations of the deletion mutants are indicated, with truncations from the amino terminus indicated as NΔX, truncations from the carboxy terminus indicated as CΔX, and internal deletions indicated as ΔN_1-N_2 (X refers to the number of amino acids deleted, N_1 indicates the location of the first deleted amino acid, and N_2 indicates the location of the last deleted amino acid). The transformation and cytotoxicity activities of all of the deletion mutants shown here with respect to LMP have been reported previously (with the exception of CΔ55 and NΔ25) (3, 10) and are indicated by +++ or −. The transformation and cytotoxicity functions of CΔ55 and NΔ25 were determined as described previously (3, 10). +++, function identical to that of LMP; −, no detectable transformation and cytyotoxic activity.

per 2×10^6 cells plated after transfection with pSV_2-LMP (10 of 10 clones [pSV_2-CΔ55 transfection] and 5 of 5 clones [pSV_2-LMP transfection] picked from agarose expressed CΔ55 and LMP, respectively). Introduction of the nontransforming mutant of LMP (10), pSV_2-Δ25-132, into BALB/c 3T3 cells resulted in the growth of one colony per 2×10^6 cells plated, and this colony was quite small (50 μm versus ≥150 μm for the pSV_2-CΔ55- and pSV_2-LMP-transfected cells). Introduction of pCMV-CΔ55 into HH514 cells resulted in 1/1,000 the number of hygromycin-resistant colonies relative to those appearing after introduction of and selection for the parental vector (in those colonies that survived selection, only expression of rearranged CΔ55 protein was observed; wild-type CΔ55 could not be detected). The cytotoxic effect of pCMV-CΔ55 was indistinguishable from the effect of introduction of pCMV-LMP into these cells. The results of these assays demonstrate that CΔ55 is indistinguishable from the LMP protein in these two functional assays.

In addition to CΔ55, another mutant of LMP (NΔ25) was constructed to determine whether truncation of the cytoplasmic amino terminus is sufficient to inactivate the biological activity of LMP; this mutant lacks the 25-residue amino-terminal cytoplasmic domain of LMP, leaving the first membrane-spanning domain intact (pCMV-NΔ25). The activity of the protein expressed from pCMV-NΔ25 was assayed for cytotoxicity in HH514 cells. The numbers of hygromycin-resistant colonies surviving selection with hygromycin following transfection with pCMV-NΔ25 were indistinguishable from those which survived selection after transfection with the nonfunctional deletion mutant pCMV-Δ25-132; all colonies examined (total of eight) expressed the NΔ25 and Δ25-132 proteins at high levels. The numbers of hygromycin-resistant colonies which survived selection with hygromycin following transfection with pCMV-LMP were 1/300 of those observed with pCMV-NΔ25 and pCMV-Δ25 132; none of the colonies examined (total of four) expressed detectable levels of LMP. Therefore, deletion of the amino-terminal cytoplasmic domain is sufficient to inactivate the biological activity (as assayed by cytotoxicity) of LMP.

Wild-type and mutant forms of LMP were studied with respect to turnover, membrane localization, and cytoskeletal association in two cell lines. Transient assays were conducted in HEp-2 cells and in BALB/c 3T3 cells (when possible), and stable assays were conducted in BALB/c 3T3 cells. LMP has been shown to function in these cell lines (2, 10); in HEp-2 cells LMP is cytotoxic, and in BALB/c 3T3 cells LMP induces both anchorage-independent growth at moderate levels of expression and cytotoxicity when expressed at high levels. To ensure that the effects observed in HEp-2 cells were not secondary to a cytotoxic effect mediated by high-level expression of LMP and its functional deletion mutant CΔ55, the experiments conducted in HEp-2 cells were also conducted in BALB/c 3T3 cells transformed by, and expressing, LMP or in parental BALB/c 3T3 cells. In addition, the use of both HEp-2 cells (a human epithelial cell line) and BALB/c 3T3 cells (a murine endothelial cell line) for these studies ensures that the findings are peculiar neither to one species nor to one cell type.

Membrane localization of LMP and deletion mutants of LMP. LMP is present in punctate patches in the plasma membrane, as detected by immunofluorescence (17, 20). The plasma membrane location of LMP and of several LMP deletion mutants has been demonstrated also by subcellular fractionation studies (3). To determine whether the membrane localization (patching) correlates with the transforming activity of LMP, the staining pattern of each of the mutant proteins was compared with that of LMP (Fig. 2). BALB/c 3T3 cells were transfected transiently with LMP expression vectors and 48 h after electroporation were fixed for immunofluorescence and stained with an affinity-purified anti-LMP antiserum that recognizes the carboxy terminus of LMP. The LMP protein is present in punctate patches in the membrane (Fig. 2A; see also Fig. 6J and L). The amino-terminal deletion mutants (Fig. 2B and C) that are inactive (NΔ43 and NΔ25) display a diffuse, nonpatched, plasma membrane staining pattern. The mutants that lack some (Δ25-132) or all (Δ27-212) of the transmembrane domains (Fig. 2D and E) also show staining patterns distinct from that of LMP. The Δ25-132 mutant (Fig. 2D) is distributed diffusely over the plasma membrane, and the deletion mutant that lacks the entire hydrophobic domain of LMP (Δ27-212; Fig. 2E) shows a cytoplasmic staining pattern. The plasma membrane staining patterns of NΔ43 and Δ25-132 and the cytoplasmic staining pattern of Δ27-212 are consistent with their localization determined by subcellular localization (3). Neither of the two mutants that have internal deletions in the

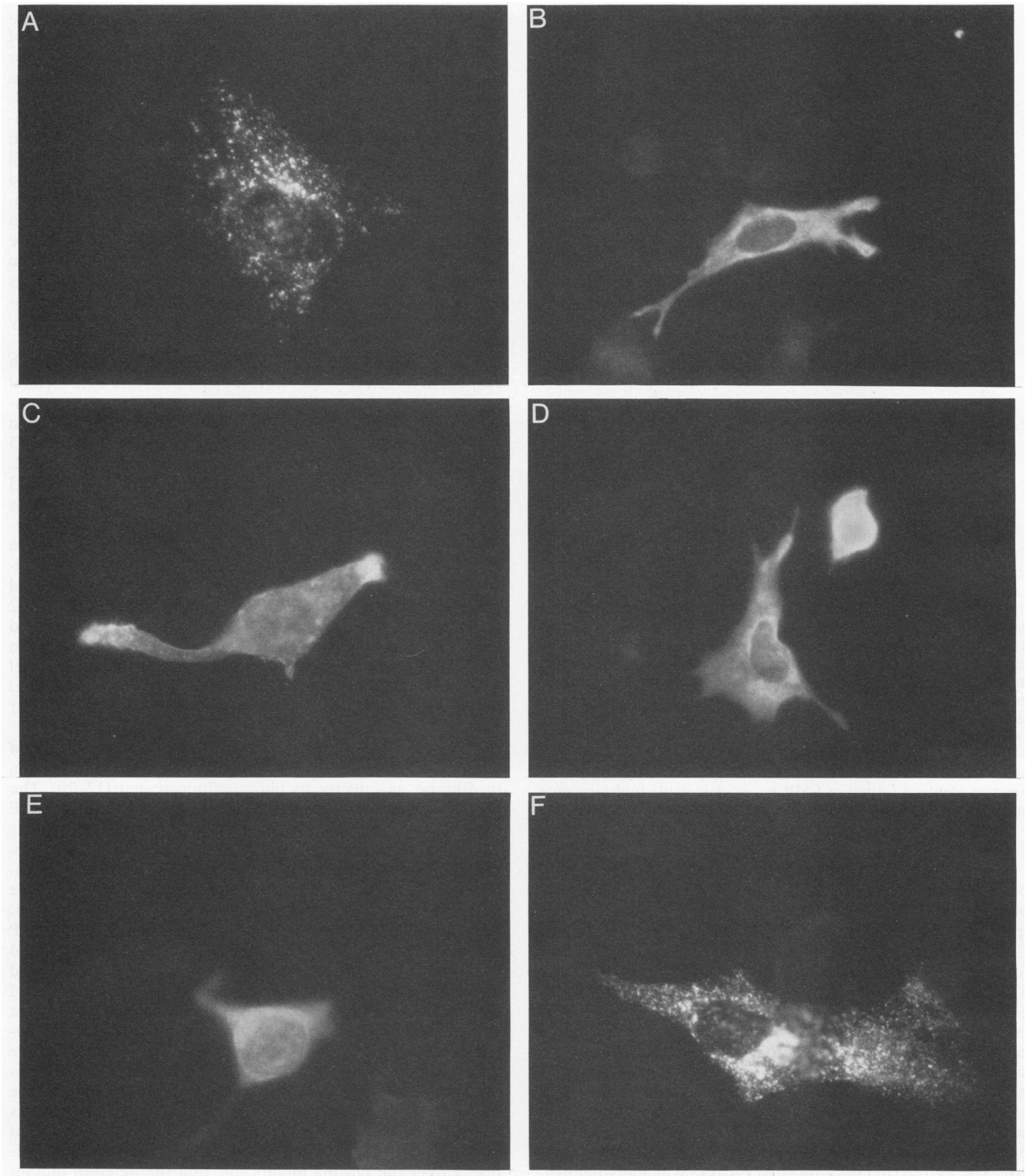


FIG. 2. Membrane localization of LMP and LMP deletion mutants in BALB/c 3T3 cells. BALB/c 3T3 cells were electroporated with pSV_2-LMP (A), pSV_2-NΔ43 (B), pSV_2-NΔ25 (C), pSV_2-Δ25-132 (D), pSV_2-Δ27-212 (E), or pSV_2-CΔ55 (F). Two days following transfection, cells were fixed for immunofluorescence and stained with an affinity-purified anti-LMP antiserum followed by a fluoresceinated second antibody as described in Materials and Methods. These staining patterns are representative of those observed in ≥50 cells scored. Magnification, ×840.

TABLE 1. LMP turnover in HEp-2 cells[a]

Plasmid	$t_{1/2}$ (h) for LMP or mutant	Function[b]
pCMV-LMP	8.3	+++
pSV_2-LMP	7.8	+++
pCMV-Δ27-212	≥24.0	−
pCMV-NΔ43	21.0	−
pSV_2-NΔ43	22.0	−
pCMV-Δ25-132	≥24.0	−
pSV_2-CΔ55	14.0	+++

[a] HEp-2 cells were transfected with the indicated plasmids; 48 h after transfection, the cells were labeled with [^{35}S]methionine-cysteine for 1 h and then chased in medium containing unlabeled methionine-cysteine for up to 24 h. At 0, 8, 18, and 24 h of chase, cells were harvested for immunoprecipitation with an affinity-purified anti-LMP antiserum. Immunoprecipitates were analyzed by SDS-PAGE and visualized by fluorography. The $t_{1/2}$s reported for LMP and mutants and those for total protein (≥24 h for all plasmids) were determined as described in Materials and Methods. The values for $t_{1/2}$ of LMP and mutant LMP proteins, and corresponding total protein turnover, represent the average values determined in three to six independent experiments, with standard deviations of 20%. The $t_{1/2}$ values for total protein (≥24 h) could not be accurately determined because the calculated $t_{1/2}$ was longer than the longest time point in the experiment.

[b] Assayed by induction of anchorage-independent growth of BALB/c 3T3 cells or cytotoxicity as described for Fig. 1. See legend to Fig. 1 for definition of symbols.

transmembrane domain of LMP retain the ability to induce anchorage-independent growth. The staining pattern observed for the CΔ55 mutant is shown in Fig. 2F. This protein is indistinguishable from LMP with respect to induction of anchorage-independent growth and cytotoxicity (see above) and similarly is present in punctate, localized patches in the plasma membrane. The results in Fig. 2 demonstrate that the membrane localization of LMP correlates with its transformation function.

Turnover of LMP and deletion mutants of LMP. LMP has a short half-life in BALB/c 3T3 cells rendered anchorage independent by the LMP gene and in EBV-positive lymphoblastoid cell lines (1, 21). To determine whether the rapid turnover of LMP was related to its capacity to induce anchorage-independent growth, two approaches were taken. First, plasmids expressing LMP or mutants of LMP from either the SV40 or the CMV promoter were transfected into HEp-2 cells in order to measure the turnover of LMP in a transient assay. HEp-2 is a human cell line that is an efficient recipient for transfection; it is easy to detect LMP expression transiently in these cells, and LMP has been shown to function in these cells (10). Two days after transfection, the half-life of LMP and its mutants was determined by pulse-chase analysis of [^{35}S]methionine/cysteine-labeled cells as described in Materials and Methods. The results of these experiments are shown in Table 1. LMP has a half-life of 8 h whether it is expressed at moderate levels from the SV40 promoter or at high levels from the CMV promoter. This turnover is shorter than the turnover of the bulk protein in the cell (half-life of 24 h) as assayed by TCA precipitation of ^{35}S-labeled cell lysates. The nonfunctional deletion mutants (Δ27-212, NΔ43, and Δ25-132) do not turn over as rapidly as LMP and, in fact, turn over with half-lives similar to that of the bulk protein in the cell. It is noteworthy that the turnover of the wild-type protein, as well as that of the representative nonfunctional deletion mutant NΔ43, is unaffected by expression at moderate levels (SV40 promoter) or high levels (CMV promoter) (Table 1). Thus, turnover rates of LMP and its mutants are independent of the promoter used to drive their expression (and therefore the level of their expression). In contrast, the CΔ55 deletion mutant, which induces anchorage-independent growth in a manner indistinguishable from LMP, has an intermediate half-life of 14 h. LMP has a three- to fourfold-longer half-life in HEp-2 cells than in BALB/c 3T3 cells. Although we do not know why LMP is more stable in HEp-2 cells, its relative stability could result from HEp-2 cells having a longer doubling time than do BALB/c 3T3 cells.

It was not possible to measure the turnover of LMP in BALB/c 3T3 cells soon after transfection. For this reason, and to compare the half-lives of mutant and wild-type LMP proteins expressed in the same cell, vectors expressing mutants of LMP were stably introduced into a derivative of BALB/c 3T3 that expresses wild-type LMP (BALB cl. 5), and the turnover of the expressed proteins was measured by pulse-chase analysis (Fig. 3A and B). LMP has a half-life shorter than that of the nonfunctional deletion mutants (Δ25-132, Δ27-212, and NΔ43); the half-life for LMP is 3.3 h, compared with 8 h for the mutants (Fig. 3A and B). This half-life (for wild-type LMP) is similar to that of LMP measured in several other independently derived LMP-transformed BALB/c 3T3 clones picked from agarose; the half-life of LMP determined in these clones ranges from 2 to 3.5 h (2). Similar results were observed in a derivative of the EBV-positive B lymphoblastoid cell line 721 that expresses both LMP and the Δ25-132 deletion mutant; LMP has a half-life of ~3 h, and the nonfunctional mutant has a half-life of ~24 h in these cells (data not shown).

To determine the half-life of the CΔ55 protein, BALB/c 3T3 cells were stably transfected with the CΔ55 gene driven by the SV40 early promoter and enhancer. Hygromycin-resistant cell lines expressing CΔ55 were analyzed by pulse-chase analysis, and the half-life of the mutant was compared with that of LMP expressed in BALB cl. 5 cells (Fig. 3C). CΔ55 has a half-life similar to that of LMP. The average half-life of CΔ55 determined from multiple determinations of two independent clones is 4.3 h, compared with 3.3 h for LMP in BALB cl. 5 (and from 2 to 3.5 h for LMP in other LMP-transformed BALB/c 3T3 clones [2]). A summary of the results of these experiments is shown in Table 2. Those mutants that do not retain biological activity as assayed by the ability to induce anchorage-independent growth or cytotoxicity also do not turn over rapidly relative to LMP, both in HEp-2 cells when assayed transiently and in BALB/c 3T3 cells when assayed stably. The CΔ55 mutant, which is indistinguishable from LMP in its capacity to induce anchorage-independent growth, has a half-life similar to that of LMP. These results indicate (i) that the amino terminus of LMP is required for both transformation and turnover and (ii) that the carboxy terminus is dispensable for transformation and may affect, but is not essential for, the turnover of LMP; truncation of the last 55 amino acids of LMP may affect the $t_{1/2}$ of CΔ55, rendering it slightly longer than that of wild-type LMP.

Cytoskeletal association of LMP and deletion mutants of LMP. LMP has been shown to associate with the cytoskeletal compartment of the cell (16, 21). In these studies, cytoskeletal association has been operationally defined as the fraction of total cellular LMP that is insoluble in 1% nonionic detergents. The association of LMP with the cytoskeleton may be essential for its transforming activity, as is likely to be the case for the oncogene v-*src*. The transforming activity of wild-type and mutant v-*src* gene products correlates with their association with Triton X-100-resistant cellular structures (9). We determined similarly whether the cytoskeletal association of wild-type and mutant forms of

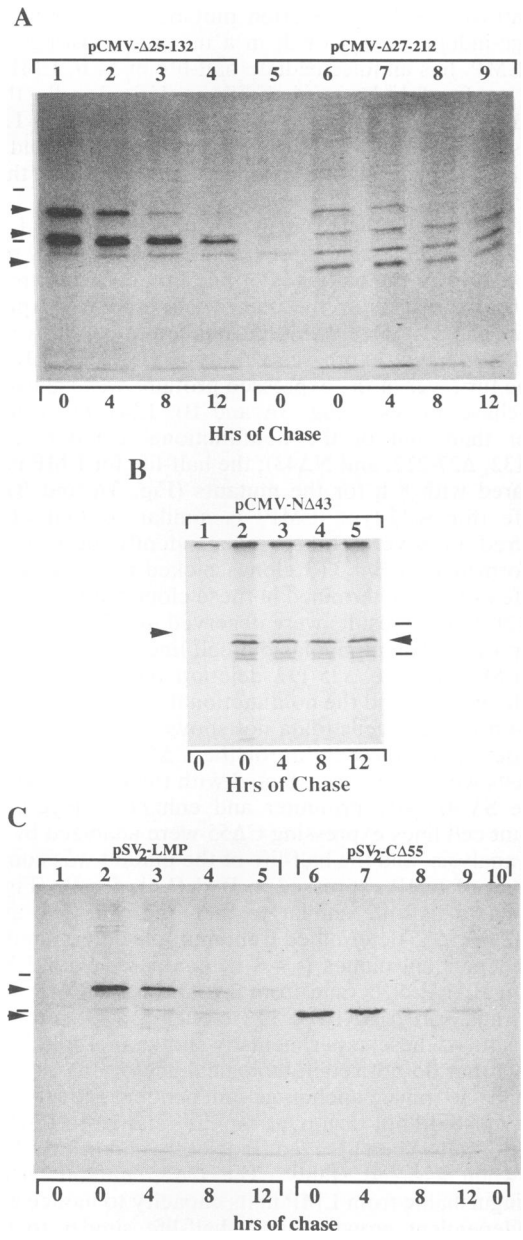


FIG. 3. Turnover of LMP and LMP deletion mutants in BALB/c 3T3 cells. BALB/c 3T3 cells stably expressing LMP and either Δ25-132 or Δ27-212 (A), LMP and NΔ43 (B), or LMP alone and CΔ55 alone (C) were labeled with [^{35}S]methionine-cysteine for 1 h and chased in medium containing unlabeled methionine-cysteine for up to 12 h. Cell extracts were prepared for immunoprecipitation with an affinity-purified anti-LMP antiserum at the indicated time points, and immunoprecipitates were analyzed by SDS-PAGE and visualized by fluorography. The duration of chase is indicated below each lane. ^{35}S-labeled extracts immunoprecipitated with preimmune serum are shown in lane 5 of panel A, lane 1 of panel B, and lanes 1 and 10 of panel C. All other lanes show extracts (equivalent to 5×10^5 cells) immunoprecipitated with anti-LMP immune serum. The transfected plasmids are indicated above each set of immunoprecipitations. The arrows in panel A point to (from top to bottom) the LMP protein, the Δ25-132 protein, and the Δ27-212 protein; the arrows in panel B point to (from top to bottom) the LMP protein and the NΔ43 protein; the arrows in panel C point to LMP and the CΔ55 protein. The bands that are not marked by arrows in panels A and C are recognized by preimmune serum and are unrelated to LMP; the bands that are not marked by arrows in panel B are degradation products derived from LMP and NΔ43, are inconsistently detected, and were generated during cell lysis or in the cell (1). Two of the size markers (68 and 43 kDa) listed in Materials and Methods are indicated on the right of the fluorograph by dashes. The half-lives of LMP and LMP deletion mutants were determined by laser densitometric scanning of fluorographs, and the half-lives of total protein were determined by TCA precipitation of ^{35}S-labeled extracts. The exposure time for this fluorograph was 48 h at −70°C. These values are shown in Table 2 and are representative of two to five independent experiments.

TABLE 2. LMP turnover in BALB/c 3T3 cells[a]

Plasmid	$t_{1/2}$ (h) for LMP or mutant	Function[b]
pSV_2-LMP	3.3	+++
pCMV-NΔ43	8.0	−
pCMV-Δ27-212	8.0	−
pCMV-Δ25-132	8.0	−
pSV_2-CΔ55	4.3	+++

[a] BALB/c 3T3 cells stably transfected with pSV_2-LMP, pSV_2-LMP plus pCMV-NΔ43, pSV_2-LMP plus pCMV-Δ27-212, pSV_2-LMP plus pCMV-Δ25-132, or pSV_2-CΔ55 were labeled with [^{35}S]methionine-cysteine for 1 h and then chased in medium containing unlabeled methionine-cysteine for up to 12 h. At 0, 4, 8, and 12 h of chase, cells were harvested for immunoprecipitation with an affinity-purified anti-LMP antiserum. Immunoprecipitates were analyzed by SDS-PAGE and visualized by fluorography. The $t_{1/2}$s reported for LMP and mutants and those for total protein (≥12 h for all plasmids) were determined as described in Materials and Methods. The value for $t_{1/2}$ of LMP (average of 3.3 h; range of 1.4 to 4.4 h) and corresponding total protein turnover represents the average $t_{1/2}$ determined from 10 independent experiments, with standard deviations of 25%. The $t_{1/2}$ of LMP was determined as the average of $t_{1/2}$s obtained from cells expressing wild-type LMP alone (BALB cl. 5) and cells expressing wild-type LMP plus mutant. The average $t_{1/2}$ of LMP in BALB cl.5 was the same as that in clones that expressed wild-type LMP plus mutant. The values for $t_{1/2}$ of NΔ43, Δ27-212, and Δ25-132 and corresponding total protein turnover represent the average $t_{1/2}$ determined from two independent experiments. The value for $t_{1/2}$ of CΔ55 and corresponding total protein turnover represents the average $t_{1/2}$ determined in two independent experiments of two independent BALB/c 3T3 clones, with standard deviations of 20%. The $t_{1/2}$ values for total protein (≥12 h) could not be accurately determined because the calculated $t_{1/2}$ was longer than the longest time point in the experiment.

[b] Assayed by induction of anchorage-independent growth of BALB/c 3T3 cells or cytotoxicity as described for Fig. 1. See legend to Fig. 1 for definition of symbols.

LMP correlates with their transforming abilities. The fraction of LMP that is resistant to repeated detergent washes is considered the Triton-insoluble fraction and can be quantitated by Western blot analysis with anti-LMP antiserum. In these experiments the intermediate filament protein, vimentin, and not the microtubule protein, tubulin, cofractionates with LMP in the Triton-insoluble fraction (data not shown), indicating that the Triton-insoluble fraction does indeed consist of at least one component of the cytoskeleton (intermediate filaments). In addition, these results indicate that the association of LMP with the cytoskeleton does not involve an interaction with tubulin and are consistent with a possible interaction with the intermediate filaments (16).

The results from fractionations of LMP in HEp-2 and BALB/c 3T3 cells with Triton X-100 are shown in Fig. 4 and 5, respectively. HEp-2 cells were transfected with the indicated expression vectors and 48 h later were fractionated as described in Materials and Methods. Approximately 25 to 40% of the total LMP expressed in these cells is Triton insoluble (Fig. 4; Table 3). In contrast, the nontransforming deletion mutants NΔ43 and Δ25-132 (see also Fig. 7 for fractionation of the nonfunctional deletion mutants NΔ25

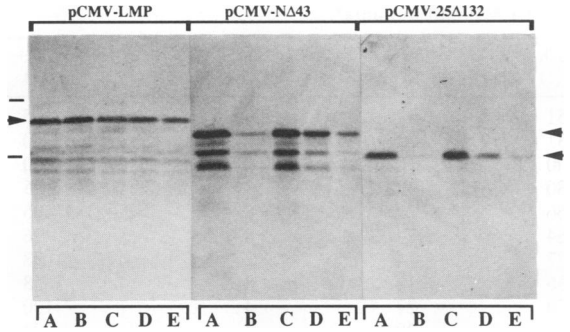


FIG. 4. Triton X-100 fractionation of LMP and LMP deletion mutants expressed transiently in HEp-2 cells. Hep-2 cells were transiently transfected with the indicated plasmids and 2 days after transfection were fractionated into Triton-soluble and Triton-insoluble fractions as described in Materials and Methods. Fractionated extracts were separated by SDS-PAGE and visualized by Western blot analysis with an affinity-purified anti-LMP antiserum. Lanes: A, unfractionated extracts; B, Triton-insoluble extracts; C, first Triton wash; D, second Triton wash; E, third Triton wash. Equal percentages of each extract (equivalent to 5×10^4 cells) were loaded on each lane and thus can be directly compared. The signals observed are within the linear range of the assay (data not shown) and were quantitated by laser densitometric scanning of blots. Values are presented in Table 3. The arrows point to the LMP, NΔ43, and Δ25-132 proteins. The markers have the same meaning as described in the legend to Fig. 3. The bands that migrate faster than LMP, and whose detection is variable, are derived from LMP (data not shown) and represent proteolysis of LMP in the cell or upon cell lysis (1).

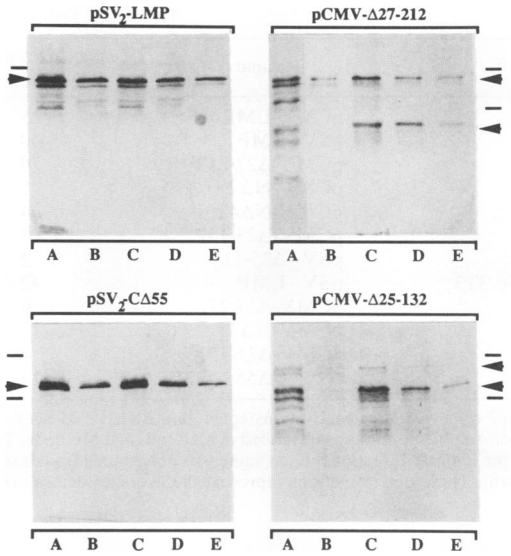


FIG. 5. Triton X-100 fractionation of LMP and LMP deletion mutants stably expressed in BALB/c 3T3 cells. BALB/c 3T3 cell lines stably expressing LMP (pSV_2-LMP), LMP plus Δ27-212 (pCMV-Δ27-212), LMP plus Δ25-132 (pCMV-Δ25-132), or CΔ55 (pSV_2-CΔ55) were fractionated into Triton-soluble and Triton-insoluble fractions as described in Materials and Methods. Extracts were separated by SDS-PAGE and visualized by Western blot analysis with an affinity-purified anti-LMP antiserum. Lanes: A, unfractionated extract; B, Triton-insoluble fraction; C, first Triton wash; D, second Triton wash; E, third Triton wash. The plasmids introduced into each cell line are indicated above each blot. Arrows indicate the location of LMP (pSV_2-LMP), LMP and Δ27-212 (pCMV-Δ27-212), LMP and Δ25-132 (pCMV-Δ25-132), and CΔ55 (pSV_2-CΔ55). Markers are indicated by dashes and have the same meaning as described in the legend to Fig. 3. Equal percentages of each extract (equivalent to 5×10^4 cells) were loaded per lane; samples within each cell line can therefore be directly compared. The signals obtained are within the linear range of the assay (data not shown) and were quantitated by laser densitometric scanning of blots. Values are presented in Table 3.

and Δ27-212) are completely soluble in Triton X-100 despite their presence in the plasma membrane. The association of LMP and the lack of association of the nonfunctional deletion mutant Δ25-132 with the cytoskeleton are independent of their levels of expression in the cell. The fractionation pattern of LMP in Triton X-100 does not vary in cells transfected with pCMV-LMP versus pSV_2-LMP, and the same is true for cells transfected with pCMV-Δ25-132 versus pSV_2-Δ25-132.

The fractionation of LMP and its mutants in BALB/c 3T3 cells is shown in Fig. 5. Cell lines that express both LMP and a nonfunctional mutant or that express only CΔ55 were fractionated as described. The results parallel those observed in HEp-2 cells (Fig. 4 and 7; Table 3) and those observed in BALB/c 3T3 clones expressing nonfunctional mutants individually in the absence of wild-type LMP (not shown). The proteins encoded by mutants that do not induce anchorage-independent growth are solubilized by washing with Triton X-100 (Fig. 5, pCMV-Δ27-212 and pCMV-Δ25-132, lanes B). The protein encoded by the CΔ55 deletion mutant induces anchorage-independent growth and is not efficiently solubilized, although it is more soluble than is LMP (Fig. 5, pSV_2-LMP and pSV_2-CΔ55, lanes B). The results of the HEp-2 and BALB/c 3T3 experiments are summarized in Table 3, and the percentage of LMP protein in each fraction determined by laser densitometry is presented. LMP, when expressed from either the SV40 promoter (moderate levels of expression) or the CMV promoter (high levels of expression) in HEp-2 cells or from the SV40 promoter in BALB/c 3T3 cells, is associated with the cytoskeleton (25 to 42%). The mutant that is functional for transformation (CΔ55) associates with the cytoskeleton, although to a lesser extent than does LMP (19%). None of the nonfunctional deletion mutants associate with the cytoskeleton (2 to 7% present in the Triton-insoluble fraction).

Relationship of the patching of LMP to its association with the cytoskeleton. To determine the nature of the operationally defined, cytoskeletally associated (Triton-insoluble) LMP, HEp-2 cells were transfected with SV_2-LMP and 48 h after transfection were fractionated into Triton-soluble and -insoluble fractions. The Triton-insoluble residue left on the plate was fixed for immunofluorescence and stained with the anti-LMP antiserum (data not shown). LMP is present in patches following extraction with 1% Triton X-100, demonstrating that the patches seen in unfractionated, fixed, and permeabilized cells represent in part that fraction of LMP that is associated with the cytoskeleton. Identical results were obtained with BALB/c 3T3 cells transfected with pSV_2-LMP (data not shown).

We perturbed the cytoskeleton by treatment with nocodazole, a tubulin-depolymerizing drug, to determine whether the patching of LMP could be dissociated from its operationally defined association with the cytoskeleton. The intermediate filament network that contributes to the cytoskeleton collapses into a perinuclear ring upon treatment with nocodazole, as a result of dissolution of the microtubules (Fig. 6E to H) (8, 30). HEp-2 cells were transfected with pSV_2-LMP, pCMV-NΔ25, or pCMVΔ27-212 and 48 h following transfection were treated for 60 min with nocodazole (50

TABLE 3. Triton X-100 fractionation of LMP and mutants[a]

Cell	Plasmid	Triton insoluble (%)	Wash 1 (%)	Wash 2 (%)	Wash 3 (%)	Washes 1 + 2 + 3 (%)
HEp-2	pCMV-LMP	25	51	17	7	75
	pSV_2-LMP	40	31	21	8	60
	pCMV-Δ27-212	0	100	0	0	100
	pCMV-NΔ25	5	80	12	3	95
	pCMV-NΔ43	4	66	24	6	96
	pCMV-Δ25-132	4	64	24	8	96
	pSV_2-Δ25-132	2	77	14	7	98
BALB/c 3T3	pSV_2-LMP	42	36	14	8	58
	pCMV-Δ27-212	0	70	27	4	100
	pCMV-NΔ43	7	66	20	7	93
	pCMV-Δ25-132	4	68	24	4	96
	pSV_2-CΔ55	19	65	12	4	81

[a] HEp-2 cells were transiently transfected, and BALB/c 3T3 cells were stably transfected, with the indicated plasmids and fractionated into Triton-insoluble and Triton-soluble fractions as described in Materials and Methods. The values in each column represent the percentage of the total unfractionated LMP or mutant LMP in the indicated fractions. Each value was determined from laser densitometric scanning of Western blots and falls within the linear range of the assay (data not shown). These determinations represent the averages determined from up to three experiments, with standard deviations of 10 to 20%.

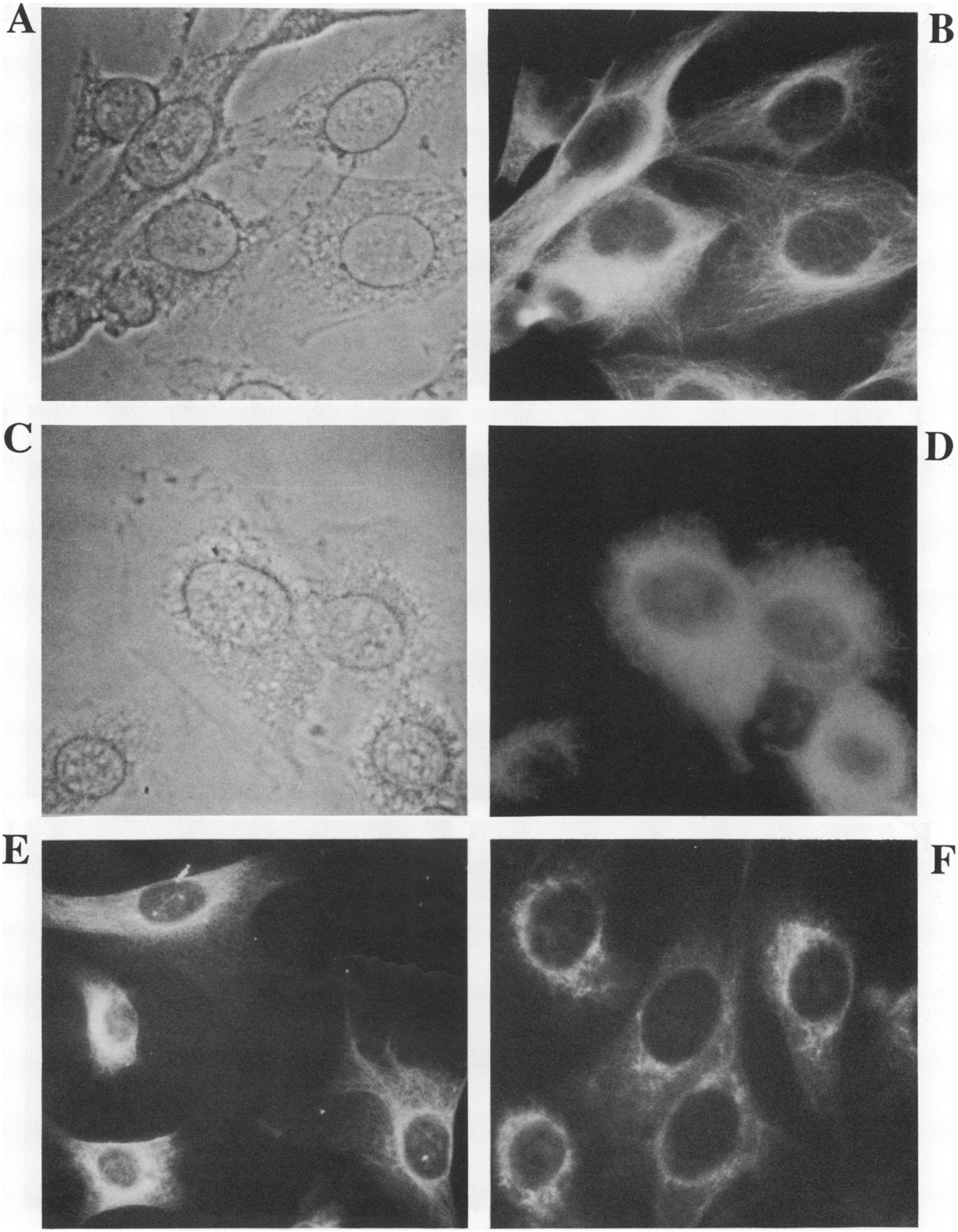
ng/ml). This treatment proved sufficient to depolymerize tubulin completely, as determined by immunofluorescence in HEp-2 cells (data not shown) and in BALB/c 3T3 cells (Fig. 6A to D). Nocodazole treatment has the same effect on LMP-transfected cells as it does on nontransfected cells with respect to collapse of the intermediate filaments around the nucleus (data not shown). LMP remained distributed in patches in transiently transfected BALB/c 3T3 cells whether or not the cells were treated with nocodazole (Fig. 6I to L). After nocodazole treatment, HEp-2 cells were fractionated in Triton X-100 as described earlier (Fig. 7). LMP is present in the Triton-insoluble fraction, while NΔ25 and Δ27-212 are not. This fractionation pattern is unaffected by treatment with nocodazole (Fig. 7, +nocodazole). The dissolution of tubulin and the collapse of the intermediate filament network affects neither the patching of LMP nor its association with the cytoskeleton. These findings indicate that these two properties of LMP measured in BALB/c 3T3 cells are probably related.

Turnover of the Triton-insoluble form of LMP. LMP has a short half-life in the cell and is associated with the cytoskeleton. It is possible that only the fraction of LMP that associates with the cytoskeleton (or vice versa) exhibits the property of rapid turnover. The half-lives of Triton-soluble and Triton-insoluble LMP in transfected HEp-2 cells and in BALB/c 3T3 cells stably expressing wild-type LMP (BALB cl. 5) were therefore measured. Cells were pulsed with [^{35}S]methionine/cysteine for 1 h and then chased in medium containing unlabeled methionine/cysteine for up to 24 h. At each time point, cells were fractionated into Triton-soluble and -insoluble fractions as already described. These fractions were immunoprecipitated with the anti-LMP antiserum and separated by gel electrophoresis. The half-lives of the Triton-soluble, Triton-insoluble, and unfractionated LMP in BALB cl. 5 cells are 3.1, 4.0, and 3.3 h, respectively (Table 4). In addition, there is no difference in the half-life (8 h) of LMP in unfractionated, Triton-insoluble, or Triton-soluble fractions measured in HEp-2 cells (not shown). This observation demonstrates that the turnover of LMP is independent of its cytoskeletal association. The reason for the apparent discrepancy between these results and those of Mann and Thorley-Lawson (21) is unclear but may result in part from differences in solubilization methods; both the detergents used and the method for solubilization were different. It is important to note that the results reported here are internally consistent; the half-lives of the Triton-soluble, Triton-insoluble, and total LMP do not differ significantly (Table 4).

DISCUSSION

Mutants of the LMP gene of EBV have been analyzed to identify the biochemical properties of the encoded derivatives of LMP that correlate with the effects of these forms of LMP on cell growth. One mutant, CΔ55, behaves like the wild-type LMP; both transform BALB/c 3T3 cells to anchorage-independent growth, and both are toxic to all cell lines tested when expressed at high levels. Four other mutants do not affect cell growth. These six forms of LMP were tested for three biochemical properties: (i) the rate of turnover of each of the proteins, (ii) their ability to patch in the plasma membrane, and (iii) their association with the cytoskeleton. LMP and CΔ55 have short half-lives, are patched in the membrane, and are associated with the cytoskeleton. The four mutants that fail to affect cell growth turn over slowly, do not patch, and do not associate with the cytoskeleton. Each of these three biochemical properties of LMP, therefore, correlates with LMP's effects on cell growth (Table 5). The correlation between the function of LMP and its cytoskeletal association and membrane localization reported here confirms and extends the results obtained for the naturally occurring truncated form of LMP (28, 29).

FIG. 6. Effect of nocodazole on the membrane localization of LMP. BALB/c 3T3 cells were treated with (C, D, G, H, K, and L) or without (A, B, E, F, I, and J) nocodazole (50 ng/ml) for 30 min at 37°C, fixed for immunofluorescence, and stained with antitubulin monoclonal antibodies (B and D), antivimentin antisera (E, F, and H), or affinity-purified anti-LMP antisera (J and L). The cells shown in panels I to L were transfected transiently with pSV_2-LMP 2 days prior to nocodazole treatment. The membrane localization of LMP was independent of nocodazole treatment; no change in LMP staining pattern was observed upon treatment with nocodazole (>100 LMP-expressing cells scored). The photographs shown in panels A, C, G, I, and K represent phase-contrast images of the corresponding stained cells in panels B, D, H, J, and L. Magnifications: A to D and F to L, ×920; E, ×580.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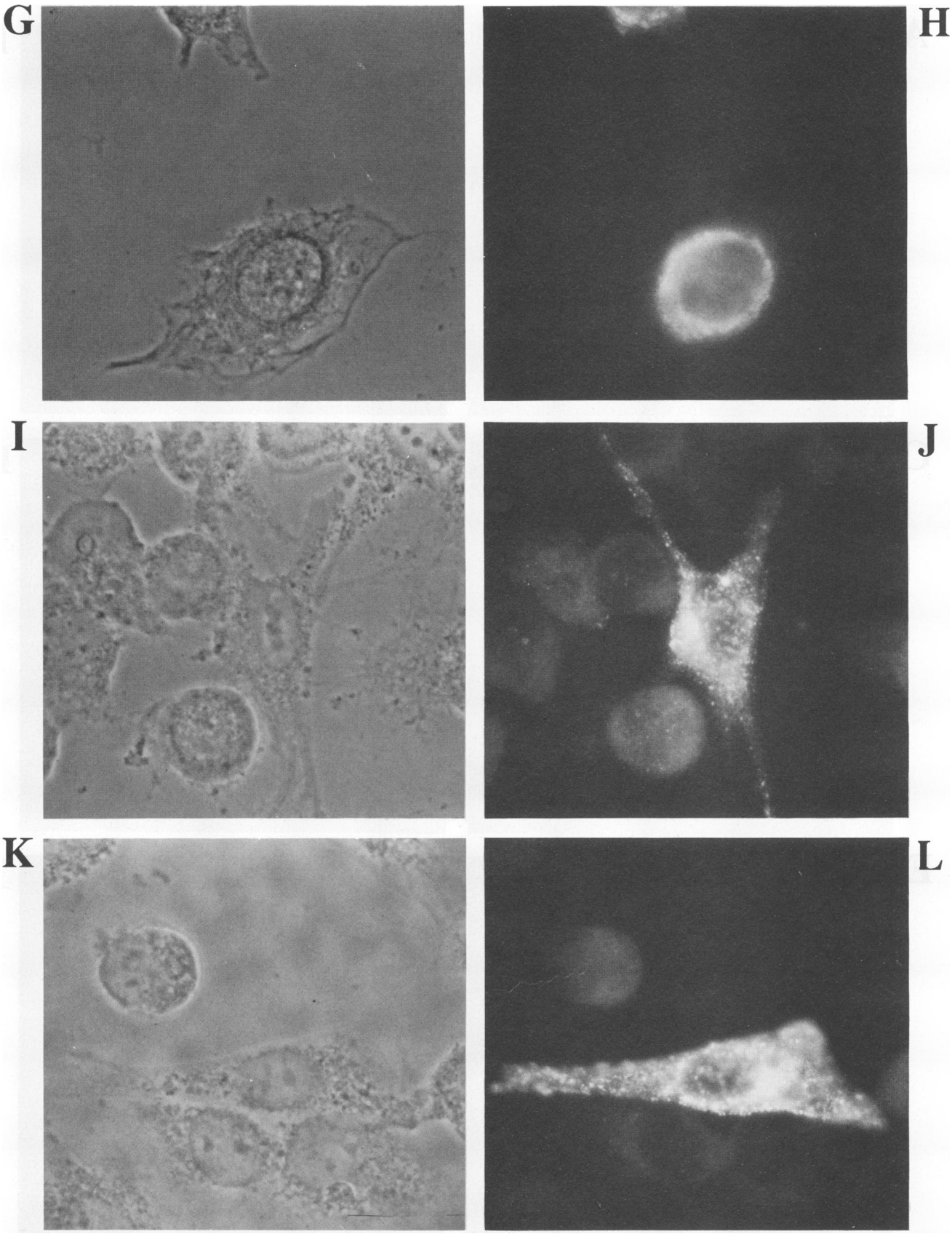

FIG. 6—*Continued.*

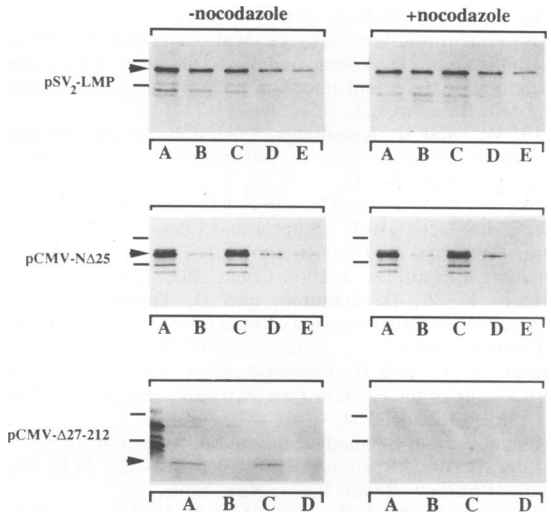

FIG. 7. Effect of nocodazole on the Triton X-100 solubility of LMP in HEp-2 cells. HEp-2 cells were transiently transfected with pSV_2-LMP, pCMV-NΔ25, and pCMV-Δ27-212 and either not treated or, 48 h after transfection, treated with nocodazole (50 ng/ml) for 1 h, as indicated. Intact cells were then fractionated into Triton-soluble and Triton-insoluble fractions as described in Materials and Methods, and extracts were separated by SDS-PAGE and visualized by Western blot analysis with an affinity-purified anti-LMP antiserum. Lanes: A, unfractionated lysate; B, Triton-insoluble fraction; C, first Triton wash; D, second Triton wash; E, third Triton wash. The signals obtained were within the linear range of the assay (data not shown) and represent the equivalent of 5×10^4 cells per lane. Arrows indicate locations of LMP or LMP deletion mutants, and dashes indicate positions of 68- and 43-kDa markers. The bands in the lane to the left of lane A in the pCMV-Δ27-212 blot (−nocodazole) represent LMP from HEp-2 cells transfected with pSV_2-LMP.

We analyzed these biochemical properties of LMP to ascertain whether they were related or independent. Two different analyses indicate that patching of LMP in the plasma membrane and its cytoskeletal association are probably related in BALB/c 3T3 cells and might reflect different facets of one property of LMP. That portion of LMP associated with the cytoskeleton is patched (not shown); disruption of polymerized microtubules and collapse of intermediate filaments affects neither patching of LMP (Fig. 6) nor its association with the cytoskeleton (Fig. 7). However, in Daudi cells LMP is patched but not associated with the cytoskeleton (15). This latter observation indicates either that patching and cytoskeletal association of LMP are generally unrelated properties or that LMP behaves peculiarly in Daudi cells. In light of the varying reports concerning the biological significance of the possible association of LMP with vimentin (this report; 15, 16, 28), the role of vimentin in the association of LMP with the cytoskeleton remains uncertain. The inability of nocodazole to alter the patching and cytoskeletal association of LMP (Fig. 6 and 7) is not consistent with LMP's being associated specifically with the intermediate filament protein vimentin but is consistent with its being associated with another cytoskeletal component such as actin filaments, as has been found for the epidermal growth factor receptor (31). The results of the experiment shown in Table 4 indicate that the properties of rapid turnover and cytoskeletal association of LMP are clearly independent: the fraction of LMP that is soluble in Triton X-100 turns over at the same rate as that which is insoluble in this detergent.

TABLE 4. Turnover of LMP in fractionated BALB/c 3T3 cells[a]

Treatment	$t_{1/2}$ (h ± SD)	n
Unfractionated	3.3 ± 1.1	10
Triton soluble	3.1 ± 0.9	5
Triton insoluble	4.0 ± 0.9	5

[a] BALB cl. 5 cells were labeled with [^{35}S]methionine-cysteine for 1 h and then chased in medium containing unlabeled methionine-cysteine for up to 24 h. Time points were taken at 0, 4, 8, 12, and 24 h, and at each time point cells were fractionated in 1% Triton X-100 as described in Materials and Methods. Fractionated samples were immunoprecipitated with an anti-LMP antiserum, analyzed by SDS-PAGE, and visualized by fluorography. The $t_{1/2}$s reported were determined as described in Materials and Methods. *n*, number of independent experiments.

TABLE 5. Summary

Protein	Structure	Function[a,b]	Turnover[b]	Membrane localization[b]	Cytoskeletal association[b]
LMP		+++	+++	+++	+++
NΔ43		−	−	−	−
NΔ25		−	−	−	−
Δ27-212		−	−	−	−
Δ25-132		−	−	−	−
CΔ55		+++	++	+++	++

[a] Assayed by induction of anchorage-independent growth of BALB/c 3T3 cells or cytotoxicity as described for Fig. 4.

[b] +++ and − are defined in the legend to Fig. 1. ++, phenotype intermediate between LMP and nonfunctional deletion mutant.

The correlation of the properties of rapid turnover, membrane patching, and cytoskeletal association of LMP with its effects on cell growth and the properties of LMP's turnover (21a) are consistent with a model in which LMP is a ligand-independent receptor. Its rapid turnover could serve to down-regulate its signaling (21a). That fraction of LMP that is patched and associated with the cytoskeleton could be poised to transduce a signal as is the patched and cytoskeletally associated form of the epidermal growth factor receptor (26) or the cytoskeletally associated form of the cyclic AMP receptor (19). If this model is accurate, signaling by LMP at one level must affect the growth requirements of some cells such that they become anchorage independent and at a higher level are lethal to a variety of cells.

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