JOURNAL OF VIROLOGY, July 1987, p. 2100–2108
0022-538X/87/072100-09$02.00/0

Vol. 61, No. 7

Posttranslational Processing of the Epstein-Barr Virus-Encoded p63/LMP Protein

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Received 19 December 1986/Accepted 18 March 1987

In this paper we describe the posttranslational processing of the p63/LMP (latent membrane protein) encoded by Epstein-Barr virus in transformed B cells. Specifically, we show that after synthesis, free LMP disappeared with a half-life of about 0.5 h. This was caused by the association of LMP with an insoluble complex. All detectable LMP in the plasma membrane was insoluble. This interaction was resistant to nondenaturing detergents but readily dissociated with 8 M urea or by boiling in 0.5% sodium dodecyl sulfate, suggesting that LMP may be associated with cytoskeletal elements. Most of the Nonidet P-40-insoluble LMP was phosphorylated (ppLMP) primarily on serine but also on threonine residues. No phosphotyrosine was detected. Furthermore, greater than 90% of the ppLMP resided in the Nonidet P-40-insoluble fraction, suggesting a strong correlation between complexing and phosphorylation. Additionally, ppLMP was found to be associated with a 53,000-molecular-weight phosphoprotein (pp53) of unknown origin. Finally, LMP turned over extremely rapidly, with a half-life of about 2 h. Taken together, these properties suggest that although LMP falls broadly within the category of phosphorylated, cytoskeleton-associated oncoproteins, it is nevertheless clearly different from any previously described member of this family.

Epstein-Barr virus (EBV) is an ubiquitous pathogen. Infection with the virus normally results in either subclinical disease or the syndrome of infectious mononucleosis (a self-limiting lymphoproliferative disease) (20). The virus infects resting human B lymphocytes in vitro and stimulates them to blast-transform (1). The cells become fixed at this stage of differentiation (44), lose growth controls, and proliferate into a lymphoblastoid cell line (EBV-LCL) (33). Such lines are growth transformed and grow indefinitely in culture but do not form tumors when injected subcutaneously into nude mice (16). The infection of the B cell, with subsequent growth transformation, is also believed to occur in vivo, and therefore the in-vitro-derived EBV-LCL is a good model system to investigate the interaction of the virus with its host.

In vitro infection by EBV has been used to investigate several questions pertaining to the mechanism of normal and neoplastic transformation of B cells. Our working hypothesis was that EBV infects and activates resting B cells by a mechanism analogous to antigen-driven activation (44). Analysis of cellular functions activated by EBV infection should, therefore, shed light on the mechanism of virus- and antigen-driven activation. Thus, we previously used the EBV system to describe the first B lineage-specific activation markers BLAST-1 (46) and BLAST-2 (44, 45), which are superinduced by EBV but also induced by other activation signals. Subsequent analysis has revealed that a shed form of the BLAST-2 molecule acts as an autocrine growth factor for a T-cell independent pathway of B-cell activation and is a tumor growth factor for EBV-transformed B cells (S. L. Swendeman and D. Thorley-Lawson, EMBO J., in press).

Similarly, analysis of the viral gene products involved in growth transformation should help in our understanding of the normal cellular pathway of activation and proliferation. EBV encodes a number of transformation-associated proteins, including at least five nuclear antigens. To date, only one nonnuclear protein has been identified. This protein is associated with membranes including the plasma membrane and has been designated LMP (latent membrane protein) (21) or p63 (31). LMP is of interest for two reasons: it has been shown to carry a target structure for EBV-specific cytotoxic T cells (D. Thorley-Lawson and E. S. Israelson, Proc. Natl. Acad. Sci. USA, in press), and it is the only EBV-encoded protein to date shown to have oncogenic potential in rodent cells (49), although the mechanism of transformation is unclear. LMP has no traditional tyrosine kinase domain and has no significant homology to any other known cellular or viral genes. This suggests that LMP is not derived from or related to any known cellular proto-oncogene and may be unrelated to any of the wide spectrum of membrane-associated transforming proteins. These include polyomavirus middle T (PmT) antigen, simian virus 40 large T antigen, and the large family of tyrosine kinase oncoproteins (2, 23) of which c-src is the prototype (8–11). We wished to discover if the LMP molecule had any of the structural features characteristic of this group of proteins and to thereby gain insight into its function. We have chosen, therefore, to study the posttranslational modification of LMP, notably association with the cellular cytoskeleton and phosphorylation, to gain insight into its functions.

MATERIALS AND METHODS

Cell lines and their maintenance. ER is an EBV-LCL derived in our laboratory by transformation with the B95-8 isolate of EBV. Jijoye is an EBV-positive Burkitt's lymphoma cell line (EBV^+ BL) obtained from G. Miller. Raji is an EBV^+ BL. BL2 is an EBV^- BL cell line obtained from G. Lenoir. All lines were maintained in 10% fetal calf serum (KC Biologicals) in RPMI 1640 (GIBCO Laboratories). Cells were passaged twice a week by diluting to 3×10^5/ml of culture supernatant.

Antibodies. The S12 monoclonal anti-LMP antibody was used throughout the study. The development of this antibody has been described previously (31). In brief, it is a

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protein-A-binding murine monoclonal antibody of the immunoglobulin G2a isotype, which is specific for an epitope in the carboxy half of the EBV membrane antigen LMP. An isotype-matched antibody was used as a negative control. The concentration or dilution of antibody and the form used (ascites or purified antibody) are described for the individual assays. Purified antibody solutions were prepared from ascites with a protein A–Sepharose C1-4b column (Sigma Chemical Co.). Elution was performed with 100 mM glycine-HCl (pH 2.5), and fractions were immediately neutralized by the addition of 4 M Tris base to the collection tubes. The fractions with the peak protein concentration were pooled, dialyzed extensively against phosphate-buffered saline, and stored at −70°C.

Radiolabeling of cells. Cells (10^7) were washed with methionine-free RPMI 1640 (GIBCO), suspended in 10 ml of 10% dialyzed fetal calf serum in methionine-free RPMI 1640 along with 0.5 to 1.0 mCi of [^{35}S]methionine (New England Nuclear Corp.), and placed in culture for 3 h. The cells were washed in RPMI 1640 and harvested for membrane preparation as described below. Radiolabeling with phosphate was performed in essentially the same manner but with phosphate-free Eagle minimal essential media (IBL Tissue Culture Products) and phosphorous 32 as P_i (New England Nuclear) for the radiolabel.

Pulse-chase analysis was performed as follows. Cells (10^7) were washed once in methionine-free RPMI 1640 (GIBCO), suspended in 10% dialyzed fetal calf serum in methionine-free RPMI 1640 with 5 to 10 mCi of [^{35}S]methionine (New England Nuclear), and incubated for 20 min at 37°C. The cells were then washed with RPMI 1640 and either harvested for membrane preparation (described below) or placed back into culture in 10% fetal calf serum in RPMI 1640 and harvested for membrane preparation after 30 or 60 min or 4 h.

Cellular fractionation and detergent solubilization. A particulate sedimentable fraction of lymphocyte lysate containing total cell membranes and hereafter referred to as the total cell membrane preparation was prepared as described previously (31). Purified plasma membranes were prepared by the method of Crumpton and Snary (13) with modification (15) as described previously (31). These samples either were used directly for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting as described below or were solubilized in a variety of detergent conditions before further analysis.

Detergents were dissolved in 10 mM Tris (pH 7.4) and included 0.5 to 1.0% Nonidet P-40 (NP-40) (Particle Data Laboratories, LTD), 1% sodium deoxycholate (Sigma Chemical Co.), 0.1% SDS (Bio-Rad Laboratories)–1.0% NP-40–1.0% sodium deoxycholate (RIPA; 11), and 1% CHAPS (Bio-Rad) (22). Where noted, detergent solutions contained EDTA (Sigma), dithiothreitol, phenylmethylsulfonyl fluoride, or NaCl. Samples were suspended in 0.2 to 1.0 ml of the appropriate detergent solution by vortexing, incubated for 30 min at 4°C with occasional vortexing, and then centrifuged at 140,000 × *g* for 30 min in the 70.1 Ti rotor of a Beckman L5-65 ultracentrifuge at 4°C to remove particulate material. Detergent-soluble material was found in the supernatant, and the pellet contained detergent-insoluble material. Soluble and insoluble fractions were analyzed by SDS-PAGE and Western blotting. In addition, NP-40-soluble material was used for immunoprecipitation and two-dimensional gel electrophoresis (described below). Alternatively, total membrane samples or insoluble fractions were prepared for immunoprecipitation by being boiled in 0.5% SDS in 10 mM Tris (pH 7.4). To reduce the concentration of SDS before immunoprecipitation, the samples were diluted fivefold with 0.5% NP-40 in 10 mM Tris (pH 7.4)–1 mM $MgCl_2$.

Immunoprecipitation. *Staphylococcus aureus* (IgGsorb, Enzyme Center Inc.) was reconstituted as directed by the supplier, and immunoprecipitation was performed essentially by the method of Kessler (26). The detergent-soluble fraction of total membrane preparations from 5×10^6 to 10×10^6 cells was prepared as described above in a volume of 0.5 to 1.0 ml. Before immunoprecipitation, extracts were precleared as follows. Isotype-matched negative control monoclonal antibody (2 μl) was added, and the sample was incubated 1 to 2 h at 4°C. Washed IgGsorb (200 μl) was added, and the sample was incubated for 1 h with vortexing at 4°C. The sample was centrifuged at 13,000 × *g* for 1 min in an Eppendorf microfuge, and the supernatant was removed to a clean tube. Another 5 μl of control ascites was added to the sample, which was incubated overnight at 4°C. IgGsorb was added, the material was centrifuged as before, and the supernatant was retained.

For specific immunoprecipitation, 10 μl of a 1:10 dilution of ascites was added to the supernatant and incubated for 4 h at 4°C. IgGsorb (50 μl) was added, and the material was centrifuged as above after a 10-min incubation. The pellet was washed six times by suspending it in 1 ml of buffer, vortexing, centrifuging as above, and aspirating the supernatant. The washes were performed with NET (50 mM Tris [pH 7.4], 5 mM EDTA, 150 mM NaCl) with 0.5% NP-40–10% saturated NaCl–1 mg of ovalbumin per ml twice, NET with 0.5% NP-40–5% saturated NaCl–1 mg of ovalbumin per ml twice, and NET with 0.5% NP-40 twice. Before the final pelleting, the solution was transferred to a clean Eppendorf tube. After the final wash the pellet was suspended in SDS-PAGE sample buffer and boiled for 5 min. This suspension was centrifuged as described above, and the supernatants were counted. Altogether, 10,000 to 20,000 cpm per lane was electrophoresed by SDS-PAGE.

Phosphoamino acid analysis. Samples for phosphoamino acid analysis were prepared by a modified version of the technique described by Weiner et al. (50). Cells (20×10^6) were labeled for 3 h with 10 mCi of ^{32}P. Total membranes were prepared and solubilized in SDS for immunoprecipitation with S12, and the immunoprecipitates were fractionated by SDS-PAGE as described above. The radiolabeled LMP was located in the gel by measuring radioactivity in sequential 2-mm slices. The labeled polypeptide was then eluted by incubation of the chopped up gel slices overnight at room temperature in 0.5 ml of 0.5% $NaHCO_3$–1 mM dithiothreitol–0.1% SDS (wt/vol) (pH 7.0). The acrylamide pieces were removed by filtration through glass wool, and the protein was precipitated by the addition of 9 volumes of acetone. The pelleted precipitate was washed three times with acetone and then dissolved in and dialyzed against two changes of 0.5% (wt/vol) ammonium bicarbonate before lyophilization. The lyophilized protein was then hydrolyzed for 2 h at 110°C in 6 N HCl under nitrogen. Phosphoamino acids were resolved and analyzed as described by Hunter and Sefton (24) by thin-layer chromatography in one dimension and high-voltage electrophoresis in the second dimension.

Western blotting. Samples were prepared in the presence of 5% 2-mercaptoethanol (Sigma) for SDS-PAGE on 10% polyacrylamide gels by the method of Laemmli (28). Molecular weights were determined by the following ^{14}C-labeled standards (New England Nuclear): myosin, 200,000;

phosphorylase *b*, 97,400; bovine serum albumin, 69,000; ovalbumin, 46,000; and carbonic anhydrase, 30,000.

Western blotting was performed by the method of Towbin et al. (47) as described previously (31) by using protein-A-purified S12 monoclonal antibody or an isotype-matched negative control (final concentrations, 0.52 μg/ml).

Two-dimensional electrophoresis and isoelectric focusing. Two-dimensional electrophoresis was performed either by the method of O'Farrell et al. (34) or the method of Singer et al. (43) as modified by Shackelford and Strominger (42). The former method was performed as follows. Gel solution containing 8 M ultrapure urea (Bio-Rad), 2% NP-40 (wt/vol), 4% ampholines (LKB Instruments, Inc.), and 4% acrylamide (Bio-Rad) was loaded into tubes (125 by 5 mm) and overlaid with 8 M urea. Samples were prepared by extracting total membrane preparations with the appropriate detergent solution as described above. The final sample buffer was adjusted to 8 M urea and 1% ampholines before being loaded onto the gels. One gel was reserved for determination of the pH gradient. The focusing was performed for 6,000 V. h. Gels containing samples were extruded from tubes and soaked for 30 min in SDS-PAGE sample buffer. The remaining gel was extruded and cut into 5-mm slices, and each slice was soaked in 0.2 ml of distilled water for pH determination. A standard 10% polyacrylamide gel was poured, with a stacking gel containing 1% agarose, and the tube gels were embedded into the liquid agarose before it solidified. SDS-PAGE and Western blotting were performed as usual.

Two-dimensional gel electrophoresis and isoelectric focusing by the method of Singer et al. (43) were as follows. Standard SDS-PAGE was performed with prestained molecular weight standards (Bethesda Research Laboratories). The gel slice corresponding to a mobility of 55,000 to 68,000 was cut out, turned 90°, and laid onto a slab isoelectric focusing gel with the same composition as described above. Isoelectric focusing was performed overnight on an LKB 2117 Multiphor II electrophoresis unit at 1,000 V. The gel was soaked for 5 min in SDS-PAGE running buffer and was Western blotted as above except that electrophoretic transfer to nitrocellulose was performed at 100 mA for 20 min.

RESULTS

Pulse-chase analysis of LMP. To gain some initial inights into the posttranslational processing of LMP/p63, we performed pulse-chase experiments. Cells were metabolically labeled with [^{35}S]methionine for 20 min and then placed into nonradioactive medium to be harvested at various times for analysis. Samples were prepared for immunoprecipitation by solubilization in SDS at 100°C or NP-40 on ice, as described in Materials and Methods. This experiment yielded two significant results: no change in mobility was observed throughout the chase (data not shown), and the amount of LMP precipitated decreased steadily with time. SDS-soluble LMP (total LMP) disappeared quickly, with a half-life of about 2 h (Fig. 1). By comparison, when the experiment was performed with NP-40 to extract LMP (free LMP), the amount of material immunoprecipitated decreased even more rapidly over h 1 (half-life about 0.5 h) and then appeared to level off (Fig. 1). The discrepancy between the fraction of labeled LMP precipitated in the presence of SDS versus that with NP-40, which was most pronounced at the 1-h chase point, indicates that most of the LMP entered an NP-40-insoluble pool as it matured. This material may, however, still be solubilized by being boiled in SDS, indicating that it has not lost the epitope recognized by S12 or become covalently attached to other insoluble moieties.

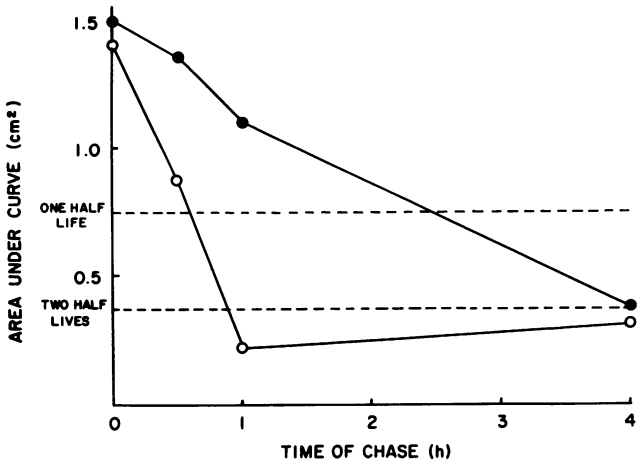

FIG. 1. Immunoprecipitation of NP-40 (○)- or SDS-plus-heat (●)-soluble LMP after pulse-labeling with [^{35}S]methionine. The EBV$^+$ BL Jijoye was pulsed with [^{35}S]methionine for 20 min and harvested for membrane preparation after chasing with nonradioactive medium for 0, 0.5, 1, or 4 h. Immunoprecipitation was performed with either the S12 monoclonal antibody or an isotype-matched negative control, and samples were analyzed by SDS-PAGE and fluorography. The specific LMP bands on the film were scanned with a densitometer, and the relative intensities were expressed as the areas under the curve of the scan.

Solubility characteristics of LMP. As discussed above, LMP appears to become NP-40 insoluble as it matures. We wished to investigate the nature of this phenomenon; therefore, we tested a variety of conditions for their ability to solubilize LMP from either an LCL line or an EBV$^+$ BL line. Similar results were obtained for both. Whole-membrane fractions were extracted with various solutions and then centrifuged to generate soluble and insoluble fractions. These fractions were then prepared for Western blot analysis by being boiled in the presence of SDS. As expected, some LMP was solubilized by NP-40 (Fig. 2, lane h), but the bulk (about 70%) was not (lane g). We have attempted LMP extraction under a variety of conditions, including the use of stronger detergents such as 1% deoxycholate (19), RIPA (1% NP-40, 1% sodium deoxycholate, 0.1% SDS) (11), and the zwitterionic detergent CHAPS (22), and the use of other additions, including EDTA, dithiothreitol, and up to 2.0 M NaCl. The bulk of LMP was insoluble under all conditions. A representative experiment is shown in Fig. 2. NP-40-soluble (lane f) and -insoluble (lane g) fractions and CHAPS-soluble (lane c) and -insoluble (lane b) fractions are shown. In this experiment approximately two-thirds of the LMP was again insoluble in either NP-40 or CHAPS. In addition, extraction of the NP-40-insoluble fraction with CHAPS (lanes d and e) did not solubilize any additional material. The double extraction was also performed under all the conditions described above, and insoluble LMP was never solubilized. We conclude, therefore, that the same fraction of LMP molecules was insoluble under all conditions tested. It is known that urea is effective in dissociating protein-protein interactions; we therefore performed detergent extractions in the presence of 8 M urea before Western blotting. The addition of urea to the NP-40-containing solubilization buffer allowed virtually all of the LMP to be solubilized (Fig. 3; compare lanes a and c and lanes b and d). In addition, if NP-40-insoluble material was reextracted in the presence of urea, most of it was solubilized (compare lanes e and f).

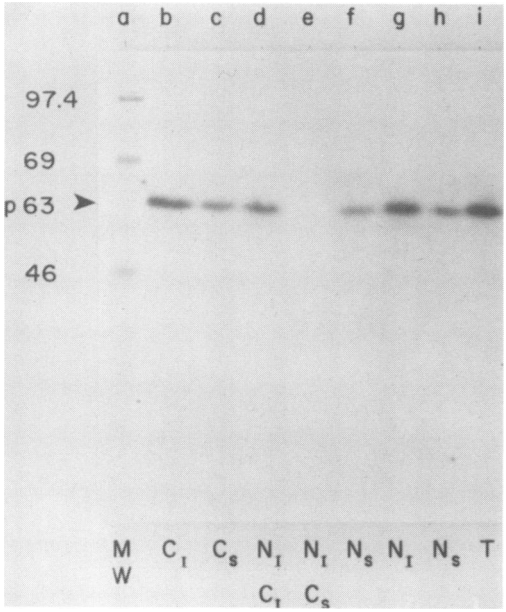

FIG. 2. Insolubleness of a fraction of LMP molecules in nondenaturing detergents. Total membranes (T) were prepared from the Jijoye cell line and were extracted with either NP-40 (N)- or CHAPS (C)-containing buffers. Soluble (N_S or C_S) and insoluble (N_I or C_I) material was analyzed by Western blotting with S12 (lanes b to i) or an isotype-matched monoclonal antibody (no reaction was seen; data not shown). NP-40-insoluble material (N_I) was reextracted with CHAPS-containing solution, and soluble (N_I C_S) and insoluble (N_I C_I) material was also anlayzed. Molecular weight standards (MW) are indicated (lane a).

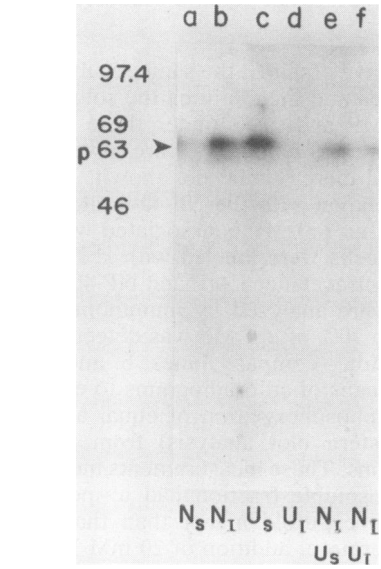

FIG. 3. Detergent-insoluble LMP solubilized with urea. Total membranes were extracted with NP-40 (N) or 8 M urea–NP-40 (U). Soluble (S) and insoluble (I) fractions are designated by subscripts. The NP-40-insoluble fraction was also extracted with urea to give soluble (N_I U_S) and insoluble (N_I U_I) fractions. All fractions were analyzed by Western blotting with S12 (lanes a to f) or an isotype-matched negative control (not shown). No reactivity was seen with the negative control.

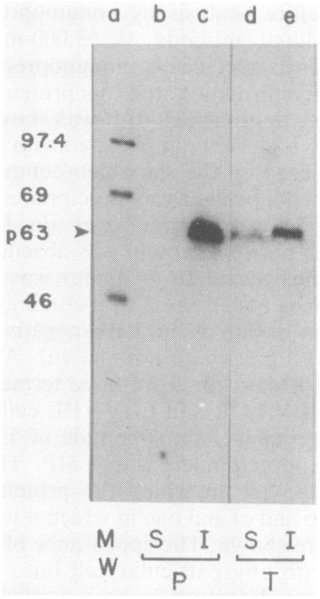

FIG. 4. Variation of LMP solubility characteristics with respect to intracellular location. Western blot analysis was performed with highly purified plasma membrane (P) and pooled intracellular membranes (T). NP-40-soluble (S) and -insoluble (I) fractions were analyzed by Western blotting with S12 (lanes b to e) or an isotype-matched negative control (not shown). No reactivity was seen with the negative control. The soluble and insoluble fractions were prepared from equal amounts of plasma or intracellular membranes. Molecular weight standards are indicated (lane a).

Since urea plus NP-40 alone was sufficient to solubilize LMP, we may conclude that the interaction that causes it to become insoluble does not involve salt bridges or disulfide bonds.

Because LMP is not intrinsically insoluble in NP-40, we conclude that as it matures, LMP becomes involved in protein-protein interactions which render it insoluble in nondenaturing conditions. Since all LMP in the plasma membrane may be considered mature, this conclusion leads to the prediction that all plasma membrane-associated LMP should be NP-40 insoluble. To determine whether the solubility of LMP was reflected in its intracellular location, subcellular fractionation was performed and the different fractions were extracted with NP-40 and analyzed by Western blotting (Fig. 4). All of the plasma membrane material was insoluble in NP-40 (compare lanes b and c), whereas NP-40-soluble material was detected only in the intracellular membrane fraction (lane d). Although plasma membrane-associated LMP was resistant to nondenaturing detergents, it was again readily solubilized upon addition of 8 M urea (data not shown). We conclude therefore that as LMP matures and passes into the plasma membrane, it becomes associated with a detergent-insoluble protein matrix. In previous studies such behavior has been taken to reflect an association with the cellular cytoskeleton (4, 6, 32, 36).

Determination of LMP as a phosphoprotein. The above analysis demonstrated that LMP, like other membrane-associated transforming proteins, became insoluble upon association with the plasma membrane. We wished, therefore, to discover if it shared a second common property, namely that it was a phosphoprotein. Cells were metabolically labeled with either [^{35}S]methionine or [^{32}P]P_i for 2 to 3 h, and total membranes were prepared and solubilized in

SDS or urea before analysis by immunoprecipitation with the S12 monoclonal antibody. A 63,000-molecular-weight phosphoprotein was specifically immunoprecipitated (Fig. 5, lanes b and c). Confirmation that this protein was LMP was obtained by three approaches. (i) It was shown to comigrate with LMP which had been radiolabeled with [^{35}S]methionine and electrophoresed on the same gel (compare lanes b and c). (ii) This phosphoprotein was precipitated with antibody specific for three different epitopes on LMP (data not shown). (iii) The phosphoprotein was absent from all EBV-negative cell lines tested (n = 4) but was detected in all EBV-positive lines tested (n = 5), including one derived by in vitro infection of one of the EBV-negative cell lines (the BL31/BL31-B95-8 pair; data not shown). Additionally, the phosphorylated LMP, which we have termed ppLMP, was found in both EBV-LCL and EBV$^+$ BL cell types. In most but not all experiments, a polypeptide of 53,000 molecular weight (pp53) coprecipitated with LMP. The results from two experiments, one in which the protein was detected (Fig. 5A, lanes b and c) and one in which it was not (Fig. 5B, lanes b and c) are shown. The appearance of this protein did not correlate with any particular cell line, and the factors that governed its detection in any particular experiment remain unclear. As it did not react with anti-LMP antibodies in Western blots, we conclude that it is not derived from LMP. Its possible significance will be further discussed below. Although the results (Fig. 5A) were achieved by solubilizing the preparations in SDS and then diluting out the

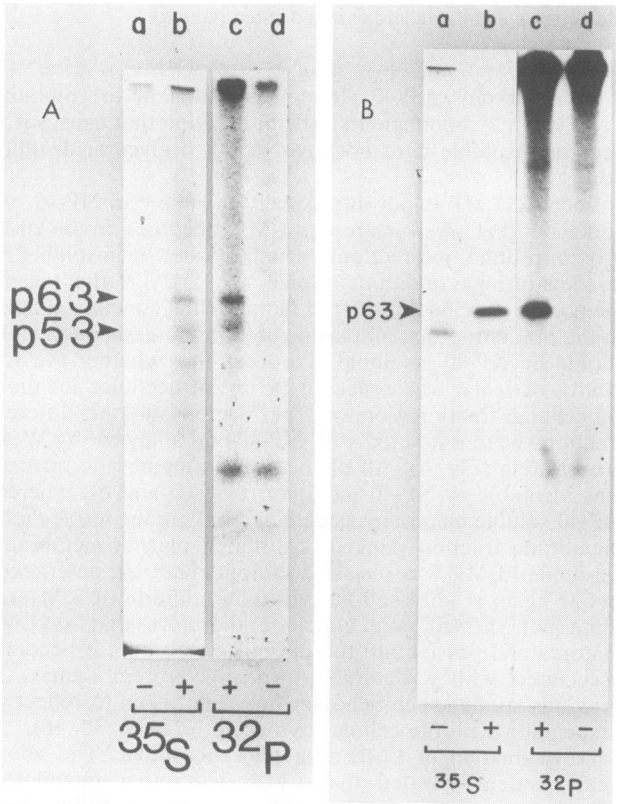

FIG. 5. LMP indicated as a phosphoprotein. Jijoye (EBV$^+$ BL) (A) and ER cells (EBV-LCL) (B) were metabolically labeled with either [^{35}S]methionine or ^{32}P. Total membranes were solubilized in SDS, and immunoprecipitation was performed with the S12 monoclonal antibody (+) or an isotype-matched negative control (−). Lanes: a and b, ^{35}S-labeled cells; c and d, ^{32}P-labeled cells.

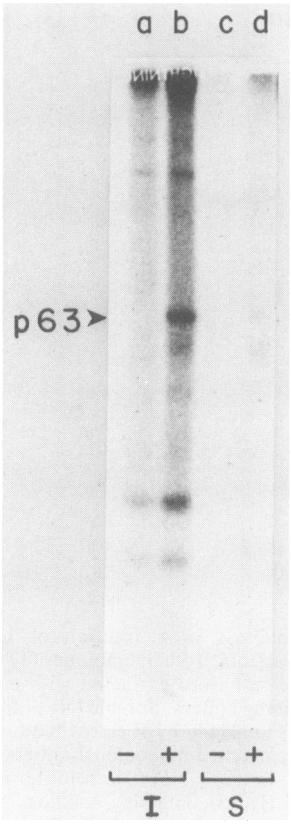

FIG. 6. Phosphorylation of detergent-insoluble LMP. Cells were labeled with ^{32}P, and the total membrane fraction was extracted with NP-40. Immunoprecipitation was performed on the NP-40-soluble (S) (lanes c and d) and -insoluble (I) (lanes a and b; solubilized on SDS) fractions with either the S12 monoclonal antibody (+) (lanes b and d) or an isotype-matched negative control (−) (lanes a and c).

detergent, we have obtained the same results by solubilizing them in 8 M urea and then diluting the solution to 2 M. We saw both ppLMP and pp53 under these conditions also; however, the immunoprecipitates are always of extremely poor quality and therefore are not shown.

ppLMP association with the NP-40-insoluble fraction. To determine whether ppLMP is associated with the insoluble LMP fraction, cells were labeled with [^{32}P]P_i for 2 to 3 h. NP-40-soluble intracellular LMP and NP-40-insoluble–SDS-soluble LMP were analyzed by immunoprecipitation (Fig. 6). Greater than 90% of ppLMP was detected in the NP-40-insoluble fraction (compare lanes b and d). We used densitometric scans of autoradiograms to estimate the relative degree of phosphorylation of equal amounts of LMP (based on Western blot analysis) from the soluble and insoluble fractions. These measurements indicated that LMP in the NP-40-insoluble fraction had a specific activity of phosphorylation eightfold higher than that in the soluble fraction. Furthermore, addition of 20 mM NaF (a phosphatase inhibitor) to the experiment did not affect the outcome. This indicates that ppLMP in the two fractions was not differentially sensitive to the action of phosphatases. We may conclude, therefore, that phosphorylated LMP correlates with the NP-40-resistant LMP.

Phosphoamino acid analysis of ppLMP. To distinguish which amino acids were derivatised, phophoamino acid analysis was performed on hydrolyzed, ^{32}P-labeled, immu-

TABLE 1. Radioactive phosphoamino acid analysis of LMP[a]

Phosphoamino acid	Radioactivity	
	cpm	Ratio
Serine	365	8
Threonine	44	1
Tyrosine	0	0

[a] Phosphoamino acid analysis was performed as described in Materials and Methods. Mixtures of radioactive and nonradioactive carrier phosphoamino acids were located by ninhydrin staining after thin-layer chromatography. The appropriate regions of the plate were scraped off and subjected to scintillation counting. A background of 37 cpm was subtracted to obtain the values shown.

noprecipitated ppLMP. The area on the thin-layer chromatography plate equivalent to each of the phosphoamino acids was identified with nonradioactive standards and scraped off, and the amount of radioactivity present was estimated. The radioactivity resided primarily in phosphoserine, with a small amount in phosphothreonine (ratio 8:1) (Table 1). No phosphotyrosine was detected.

Analysis of LMP by two-dimensional isoelectric focusing. Two-dimensional gel electrophoresis was performed for two reasons. (i) We wished to determine the pI of LMP. (ii) If LMP was associated with another structure such as pp53, the pI in nondenaturing conditions should change to reflect this association.

To determine the pI of free LMP, NP-40-soluble membranes and NP-40-insoluble membranes were solubilized by being boiled in SDS. The proteins were separated by two-dimensional gel electrophoresis with SDS-PAGE for the first dimension and isoelectric focusing with a urea–NP-40 slab gel for the second dimension. This gel was then Western blotted for visualization of LMP. The pI of both fractions was 3.8 ± 0.1 (Fig. 7A). Such a low pI was expected because of the amino acid sequence, which has a preponderance of negative over positive charged residues. In the NP-40-insoluble material, the most acidic spot (pI 3.7) was where the predominant species was detected. This species was either missing or undetectable in the NP-40-soluble material. This is consistent with the results described above, namely that the insoluble material contained ppLMP, which would be more acidic than the predominantly unphosphorylated species found in the NP-40-soluble fraction. Furthermore, the spot at pH 3.7 constituted more than half of the LMP detected in the NP-40-insoluble fraction, suggesting that most of the LMP in the plasma membrane was phosphorylated.

The predominant form of LMP found in plasma membranes was detergent-insoluble ppLMP, which was predicted to be associated with other proteins, including pp53 or the cytoskeleton. The procedure used above to determine the pI of LMP involved boiling the sample in SDS before SDS-PAGE and subsequent isoelectric focusing. This procedure totally dissociates noncovalently associated proteins, and therefore the pI does not reflect an association of LMP with other structures. It is possible, however, to perform isoelectric focusing before SDS-PAGE. The pI derived from this procedure reflects both the pI of LMP and that of any protein that remains associated in the focusing conditions. Although we knew that LMP is soluble in urea, we thought that this milder treatment might dissociate an insoluble complex but leave LMP associated with another protein. One possible candidate is the pp53 phosphoprotein, which coprecipitates with LMP.

NP-40-insoluble total membranes were prepared and reextracted with an 8 M urea–NP-40 solution. This material was electrophoresed on an isoelectric focusing tube gel for 6,000 V · h. The tube gels were then placed on a 10% polyacrylamide gel and were electrophoresed and Western blotted as described above. In this analysis, the pI of LMP was 4.5 to 4.7 (Fig. 7B). As a control, the same experiment was performed with a mixture of the NP-40-soluble and -insoluble fractions. In this case, a second spot was detected due to the NP-40-soluble fraction, with a pI of 3.8 as expected (data not shown). The pI of the NP-40-insoluble fraction is therefore clearly different from that seen with free LMP (pI 3.7 to 3.8), and this provides evidence supporting the suggestion that LMP remains associated with a cellular component in the presence of urea-containing solutions.

DISCUSSION

In this paper we have presented evidence that LMP from both EBV^+ BL and LCL shares at least two properties in common with plasma membrane-associated oncogene products: namely, it is phosphorylated (ppLMP), and it becomes associated with a detergent-resistant complex, suggesting association with the cellular cytoskeleton. All of the LMP in the plasma membrane is in such a resistant complex. Additionally, we have shown that LMP is associated with a second phosphoprotein (pp53) and has the unique characteristic of a membrane-associated transforming protein: i.e., it turns over at an extremely high rate (half-life of about 2 h).

Our analysis reveals that greater than 90% of the ppLMP was present in the detergent-resistant fraction. Additionally, we observed that most of the LMP in this fraction was phosphorylated. Phosphoamino acid analysis revealed that the phosphorylation was on serine and threonine residues. No phosphotyrosine was detected, suggesting that LMP is not a member of the tyrosine kinase family of membrane oncoproteins. This group, exemplified by pp60 src (11), includes a large number of phosphoproteins, all of which have sequence homology in their kinase domain (2, 23).

Our experiments could not distinguish whether LMP has intrinsic kinase activity; however, since LMP has no sequence homology to known protein kinases, it is most likely that LMP is phosphorylated through interaction with a cellular serine-threonine kinase. One plausible candidate is protein kinase C, which is known to be involved in the proliferation of B cells (3). Furthermore, it has been shown that during activation, the kinase is processed in vivo by specific proteolysis, yielding a 51,000-molecular-weight protein (25) with enzymatic activity. We are currently investigating whether LMP has endogenous kinase activity and whether there is a relationship between the processed form of protein kinase C and the pp53 protein that coprecipitates with LMP.

The lymphocyte cytoskeleton, like that of adherent cells, has been shown to contain an organized cell structure containing microfilaments, microtubules, and intermediate filaments (5, 32), and nonionic detergents (Triton X-100 or NP-40) solubilize the plasma membrane without disrupting the cytoskeleton (4, 6, 36). We have shown that LMP is not intrinsically insoluble in nonionic detergents, because pulse-labeled LMP and a portion of intracellular LMP were always solubilized in our study. However, free LMP became completely insoluble with a half-life of about 0.5 h, and a fraction of the insoluble form was associated with the plasma membrane. One interpretation of this observation is, therefore, that LMP shares a second common feature with membrane-associated oncoproteins, namely association with the cytoskeleton. This association represents a non-covalent

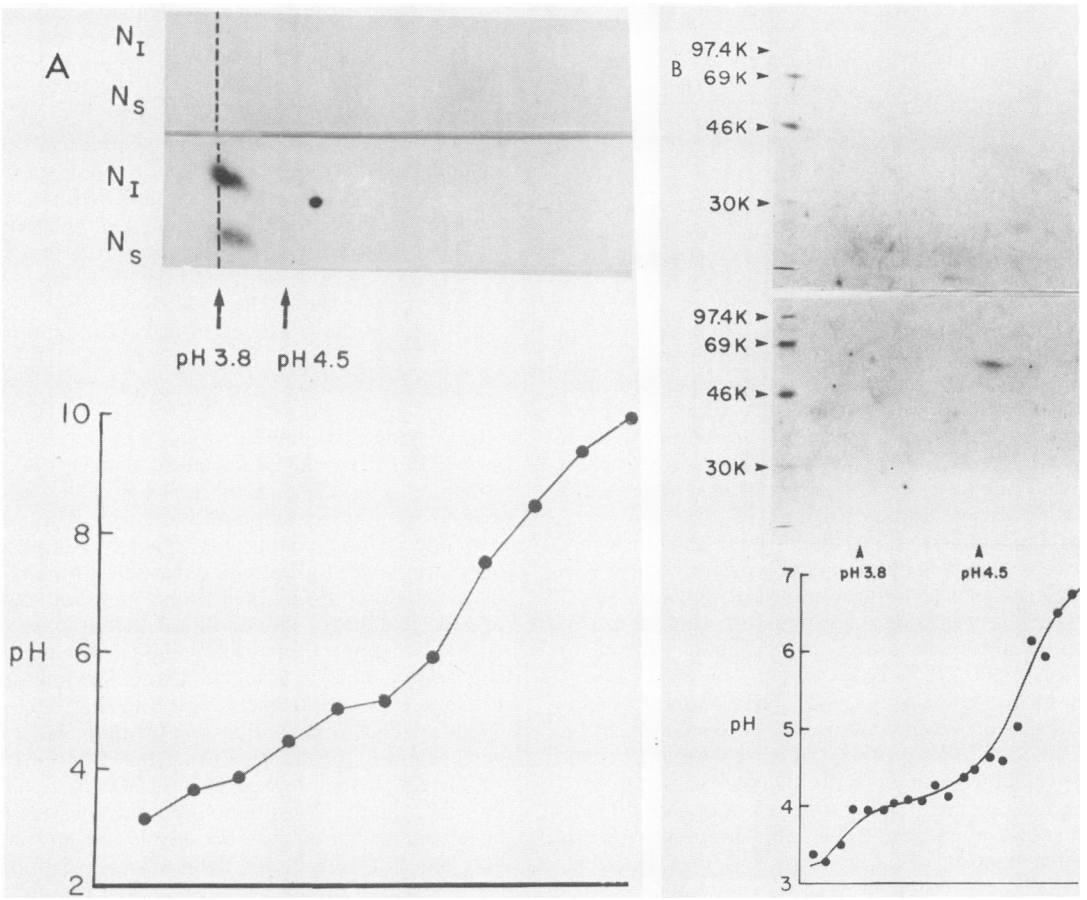

FIG. 7. (A) Two-dimensional isoelectric focusing. NP-40-soluble (N_S) and -insoluble (N_I) membranes were electrophoresed by SDS-PAGE. Gel slices were cut from each lane corresponding to mobilities of 55,000 to 68,000 molecular weight and placed on a slab isoelectric focusing gel. After focusing, the gel was Western blotted and analyzed with either the S12 monoclonal antibody (lower panel) or an isotype-matched negative control antibody (upper panel). (B) Two-dimensional isoelectric focusing of LMP. NP-40-insoluble membranes were prepared and extracted in a urea–NP-40 solution. This material was subjected to isoelectric focusing in tube gels containing NP-40 and urea. The tube gels were then overlaid onto a second gel for analysis by SDS-PAGE. Western blotting was performed with either the S12 monoclonal antibody (lower panel) or an isotype-matched negative control antibody (upper panel). Molecular weight markers are indicated on the left.

protein-protein interaction, since it was dissociated by 8 M urea or boiling in 0.5% SDS. The interaction was not disrupted by high salt conditions or thiols and, therefore, was not caused by salt bridges or disulfide bonds. The hypothesis that LMP exists in complexes is also supported by immunofluorescence studies (29, 31; unpublished observation) showing that, regardless of fixation method, LMP is visualized as large patches on the surface of the cell.

Although the putative complex became soluble after the addition of urea, two-dimensional gel electrophoresis of urea extracts indicated that at least one protein continues to associate with LMP under these conditions. A possible candidate for a molecule associating with LMP is the 53,000-molecular-weight phosphoprotein (pp53), which coprecipitated with ppLMP after solubilization in SDS or urea. This species has never been seen in Western blot analysis with anti-LMP antibodies; therefore, we conclude that it is not a degradation product of LMP. We speculate that pp53 dissociates from LMP during boiling in SDS, reassociates when the SDS is diluted, and then coprecipitates. We are uncertain of the identity of this protein; however, possible candidates include pp50, a cellular phosphoprotein that coprecipitates with the *fps*, *yes*, and *src* oncogenes (8, 30, 35); pp55, a recently described B-cell-specific phosphoprotein (14); and the 51,000-molecular-weight cleavage product of protein kinase C (25). Further analysis is necessary, however, before we can determine the identity and significance of pp53.

As discussed above, LMP bears no relationship to the tyrosine kinase family of oncoproteins. Among the membrane-associated oncoproteins, LMP seems to be most similar to PmT and simian virus 40 large T antigens since neither has intrinsic kinase activity. PmT is required for transformation (37, 41), resides in the plasma membrane, and is associated with cytoskeletal elements in a detergent-resistant interaction (40). Furthermore, most of the plasma membrane-associated PmT is phosphorylated through association with a cellular phosphokinase (c-src) (12). However, PmT is phosphorylated on tyrosine residues. Simian virus 40 large T, on the other hand, is (like LMP) phosphorylated on serine and threonine residues (18, 39) through association with a cellular kinase (48). It is also thought to associate with cytoskeletal elements in the plasma membrane (27, 38); however, only a very small fraction of total simian virus 40 large T resides in the membrane, and the relevance of this form to transformation is unclear. What distinguishes LMP

from all previously described membrane oncoproteins is its high rate of turnover. Our recent experiments suggest that the turnover of LMP is due to specific cleavage, occurring soon after phosphorylation, into a membrane-associated p35 (amino-terminal half) and a pp29 (carboxy-terminal half) that is rapidly released into the cytoplasm. Such rapid processing has not been described for other membrane-associated oncoproteins but is characteristic of proteins whose level fluctuates rapidly under tight cellular control, particularly during the cell cycle (17).

Although the function of LMP is still unclear, the current observations allow some general conclusions. We propose that LMP is a unique form of membrane-associated transforming protein that is expressed, transported to the cell surface, and then degraded with a turnover half-life of approximately 2 h. During this time it joins a high-affinity, insoluble protein complex with cytoskeletal elements and becomes phosphorylated by means of a cellular serine-threonine kinase. Our current experiments are directed toward analyzing the functional significance of this turnover, the identity of the pp53 protein, and the nature of the kinase that phosphorylates LMP.

ACKNOWLEDGMENTS

We thank Brian Schaffhausen for help in performing the phosphoamino acid analysis and helpful advice in preparing the manuscript, Cindy Welch for typing the manuscript, and Kitty Rahbany for technical assistance.

This work was supported by Public Health Service grants AI 15310 and CA 28737 from the National Institutes of Health. D.T.-L. is a recipient of a Public Health Service Research Career Development Award (grant AI 00549).

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