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

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We have analyzed the processing of the Epstein-Barr virus-encoded latent membrane protein (p63/LMP) in lymphoblastoid cell lines, Burkitt's lymphoma cell lines, and rodent fibroblasts transfected with the p63/LMP gene. Pulse-chase analysis by immunoprecipitation, under denaturing conditions, reveals a half-life of 2 h. This is due to turnover in the plasma membrane with cleavage of the protein, resulting in a 25,000-molecular-weight (p25) fragment derived from the carboxy-terminal portion of LMP. This fragment is rich in proline and acidic amino acids and sheds into the cytoplasm, where it appears to accumulate, being present in a six- to sevenfold molar excess over p63/LMP in immunoprecipitation analyses. p25 is, like p63/LMP, also phosphorylated (pp25) on serine and threonine residues, in the same ratio and to approximately the same extent as the intact p63/LMP molecule. Amino acid sequence analysis and carboxy-terminal labeling suggest that p25 is derived through a single cleavage adjacent to the sequence LGAPGGGPDNGPQDPD.

Epstein-Barr virus (EBV) infects resting human B lymphocytes (1) in vivo and in vitro and causes them to become activated and proliferate. Such virus-infected cells will grow indefinitely in culture and are referred to as immortalized or growth-transformed lymphoblastoid cell lines (EBV-LCL) (21). We have proposed that growth transformation by EBV involves two phases (25). The first phase requires the virus to activate infected B cells by a mechanism that parallels that of normal resting B cells when challenged with antigen. In the second phase the virus causes the activated B cell to become fixed at the proliferative stage of differentiation.

Recent studies indicate that virus binding alone is sufficient to trigger the initial activation of the cell (6, 13). Virally encoded nuclear proteins appear at about 24 h postinfection (14), and the cells begin to proliferate at 48 to 72 h postinfection (25). At 48 to 72 h postinfection, the only virally encoded membrane protein so far detected in growth transformed cells is found in the plasma membrane. This protein, termed p63 (19), latent membrane protein (LMP) (10), or BNLF1 (2), has a molecular weight of approximately 63,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and consists of a highly hydrophobic amino-terminal half and a highly hydrophilic, acidic carboxy-terminal half. A structure for p63/LMP in the plasma membrane has been predicted on the basis of its sequence (5, 11) (see Fig. 6C). Both amino- and carboxy-terminal sequences are predicted to be on the cytoplasmic surface and to be connected via six transmembrane sequences. This structure should expose three short reverse-turn loop sequences on the external surface. Published data are consistent with the cytoplasmic location of the carboxyl terminus (10, 19) and the external exposure of one short stretch of amino acids close to the amino terminus (16).

The function of p63/LMP is as yet unknown, although several lines of evidence suggest that it may be related to the group of membrane-associated oncoproteins and to polyomavirus middle T antigen in particular. First, p63/LMP has been shown to transform rodent fibroblasts in culture (3, 27) and to alter the phenotype of an EBV-negative Burkitt's lymphoma (EBV$^-$ BL) line (28). Second, it is phosphorylated on serine and threonine but not tyrosine residues (2, 20). A third characteristic of p63/LMP was demonstrated by pulse-chase analysis performed by immunoprecipitation with antibodies directed at the carboxy-terminal half of the protein in the presence of denaturing or nonionic detergents (2, 20). p63/LMP becomes resistant to solubilization in nonionic detergents with a half-life of approximately 30 min, owing to association with the cytoskeletal matrix (17, 20). This form of the molecule may be readily solubilized, however, with urea or SDS (19). Under these conditions the protein can be shown to turn over in the membrane at a high rate, with a half-life of 2 to 3 h (2, 20).

In the present study we present evidence that the turnover is due to the specific release into the cytoplasm of a 25,000-molecular-weight carboxy-terminal fragment of the protein.

MATERIALS AND METHODS

Cells and antibodies. JY, ER, and RKM are EBV-LCLs derived in our laboratory by transformation with the B95-8 isolate of EBV. Jijoye is an EBV$^+$ BL obtained from G. Miller. The EBV$^-$ BL BL-1-2-31 and the EBV$^+$ BL BL-36 were the kind gift of G. Lenoir. The EBV$^+$ BL cell lines AG876 and RAJI, the EBV$^-$ BL cell line RAMOS, the erythroleukemia line K562, and the murine fibroblastic line BALB/c 3T3 were all obtained from the American Type Culture Collection. NIH 3T3 cells were the kind gift of T. Krontiris. Rat-1 cells were the kind gift of Robert Weinberg.

The S12, CS-2, CS-3, and CS-4 monoclonal antibodies are murine monoclonal antibodies raised against a fusion protein between β-galactosidase and the carboxyl half of LMP (10). The S12 monoclonal antibody has been characterized and described in detail previously (19). The other antibodies were derived in a similar fashion to S12 and were the kind gift of M. Rowe (23). The S12 and isotype-matched negative control antibody (1A2) were used as protein A affinity-purified antibody in all experiments. The CS-2 and CS-3 antibodies were ascites fluids, and CS-4 was a cell culture supernatant. CS-2 and CS-3 plus CS-4 recognize two distinct epitopes on p63/LMP. The rabbit anti-mouse antiserum was described previously (24).

The rabbit heteroantisera against the amino terminus of

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p63/LMP were prepared as follows. A synthetic peptide consisting of the 15 amino-terminal amino acids of p63 (peptide N) was synthesized by Janis Young, Children's Hospital, Boston, Mass. The synthesis and purity of the peptide were confirmed by amino acid analysis. The peptide sequence included an additional carboxy-terminal cysteine for ease of coupling. The peptide was coupled to keyhole limpet hemocyanin (KLH) by the method of Liu et al. (18). KLH was dialyzed three times against 2 liters of phosphate-buffered saline. The retentate was then dialyzed twice against 1.5 liters of 10 mM phosphate buffer (pH 7.2). This material was centrifuged for 10 min at 13,000 × *g* in an Eppendorf microcentrifuge to remove particulate material. The protein concentration in the supernatant was determined by using the Bio-Rad protein assay (4), and the material was aliquoted (4 mg per aliquot) and stored at −70°C.

Conjugation of peptide to KLH was performed as follows. A 3-mg portion of maleic benzoyl succinamide ester (Sigma Chemical Co.) was added to 500 μl of dimethylformamide (Sigma). Maleic benzoyl succinamide ester solution (70 μl) was added to the KLH solution prepared as above and containing 4 mg of KLH. This was stirred for 30 min at room temperature, and the pH was adjusted to neutral if necessary. G-25 Sephadex (Sigma) was swollen as specified by the manufacturer, and a 15-ml column was poured and equilibrated by washing with 5 bed volumes of 50 mM phosphate buffer (pH 6.0). The maleic benzoyl succinamide ester-KLH solution was loaded onto the column and washed with 50 mM phosphate buffer (pH 6.0). Samples (1 ml) were collected, and the optical density at 280 nm was determined. The peak fractions were pooled and transferred to a clean screw-top vial. A 1-ml portion of a fresh 4-mg/ml solution of peptide was added, and the pH was adjusted to 7.0 to 7.5. This solution was stirred for 3 h at room temperature, aliquoted, and stored at −70°C.

Female New Zealand White rabbits were immunized subcutaneously and intramuscularly with 0.5 mg of peptide conjugate once every 2 weeks. The first two injections were in complete Freund adjuvant, and all subsequent injections were in incomplete Freund adjuvant. After three injections, rabbits were bled 7 to 10 days after each subsequent injection, and the sera were tested by enzyme-linked immunosorbent assay with a goat anti-rabbit immunoglobulin G-alkaline phosphatase (Sigma) conjugate. A prebleed from the same rabbit was used as a negative control. Positive sera were purified by first being passed over a protein A-Sepharose Cl-4b column (Sigma or Pharmacia) and eluted with 100 mM glycine hydrochloride (pH 2.5). Fractions were neutralized by the addition of 4 M Tris base to the collection tubes. The protein concentration was determined by the optical density at 280 nm, and the peak fractions were pooled and dialyzed extensively against PBS. This material was then affinity purified by using the appropriate peptide conjugated to Affi-Gel 10 as described by the manufacturer (Bio-Rad Laboratories). The flowthrough from the peptide affinity column was used as a negative control rabbit immunoglobulin.

Radiolabeling of cells. Steady-state and pulse-chase analyses were performed as described previously (20), except for steady-state labeling for which 2.5 mCi of [^{3}H]proline (108 Ci/mmol; Amersham Corp.) was used.

Cellular fractionation and detergent solubilization. Whole-cell lysates and subcellular fractions and detergent extracts of these were prepared as described previously (19, 20).

Immunoprecipitation, SDS-PAGE, and Western blots. Immunoprecipitation was performed by the method of Kessler (15) as described previously (20), with the following modifications. After being washed, the immunocomplexes were solubilized by boiling for 2 min in 100 μl of buffer containing 0.5% SDS, 10 mM Tris (pH 7.4), and 1 mM $MgCl_2$. The tubes were then centrifuged in the Eppendorf centrifuge as described above, and the supernatants were transferred to new tubes for reprecipitation. The samples were diluted fivefold with 400 μl of a buffer containing 0.5% Nonidet P-40, 10 mM Tris (pH 7.4), and 1 mM $MgCl_2$. Then 1 μl of S12 or isotype-matched, negative control monoclonal antibody (2.5 mg/ml) was added to the positive and negative control tubes, respectively, and the tubes were vortexed for 30 min at 4°C. IgG-Sorb (50 μl) was then added, and the tubes were agitated for 10 to 15 min at 4°C. The IgG-Sorb was pelleted by centrifugation as described above, and the pellet was washed three times in the buffers described above. After the final wash, the pellet was suspended in SDS-PAGE sample buffer and boiled for 2 to 5 min.

For the CS-2, CS-3, and CS-4 antibodies, indirect immunoprecipitation was required. To do this, 5 μl of rabbit anti-mouse antiserum was added to the preclearing steps, along with monoclonal antibody, and was added for 1 h after the incubation with specific antibody for the precipitation step. Otherwise, the procedure was the same as above, except that CS-2 and CS-3 were used as crude ascites (1 to 2 μl per immunoprecipitation) and CS-4 was used as a cell culture supernatant (100 μl per immunoprecipitation) and samples were not reprecipitated.

SDS-PAGE was performed as described previously (19, 20). Western immunoblotting was performed by the method of Towbin et al. (26) as described previously (19), with rabbit anti-N immunoglobulin and negative rabbit immunoglobulin as described above or S12 and an isotype-matched negative control (1:300 dilution).

Samples were eluted from 1-cm^2 gel slices for reprecipitation by agitation overnight at 4°C in 1 ml of buffer containing 0.5% Nonidet P-40, 10 mM Tris (pH 7.4), and 1 mM $MgCl_2$. The gel slices were then discarded, and immunoprecipitation was performed as described above.

Transfections. NIH 3T3 fibroblasts were transfected with the pSV2gpt LMP plasmid (a kind gift of E. Kieff), which contains the p63/LMP-encoding sequence under the control of the metallothionein promoter (27), or the pSV2gpt (22) control plasmid, either by the $CaCl_2$ method of Graham and Van der Eb (7) (1 to 10 μg of plasmid per 100-mm dish; 20 μg of salmon sperm DNA as carrier) or by electroporation (20 μg of plasmid DNA per 3 × 10^6 cells, 250-V pulse, and 20 μg of salmon sperm carrier DNA) with a Cellzap II Electroporator (Anderson Electronics) and Bio-Rad Gene Pulser cuvettes.

Purification of p25. A 20-liter sample of the Jijoye cell line (yielding a cell pellet of ca. 30 g) was obtained from Don Giard, Massachusetts Institute of Technology Cell Culture Center. Cell fractionation was performed as described previously (19, 20). The cytoplasmic fraction was used to purify p25.

p25 was affinity purified over an S12-monoclonal antibody column as follows. S12 and 1A2 (isotype-matched negative control) were separately linked to Affi-Gel 10 as specified by the manufacturer at an antibody concentration of 2 to 5 mg/ml of Affi-Gel. The cytoplasmic fraction of the Jijoye lysate was passed over a 20-ml Sepharose 4B column connected to a 5-ml 1A2 column. The flowthrough was then passed over a 2.5-ml S12 column. The S12 column was then

washed with 5 volumes of PBS, 5 volumes of PBS–10% NaCl, and 5 volumes of PBS.

The protein was eluted from the S12 column with 50 mM diethylamine (pH 11.5), and 15 1-ml fractions were collected. A 5-μl portion of each fraction was labeled with ^{125}I by the chloramine-T method and analyzed by SDS-PAGE. The fractions containing p25 were identified by exposure of the dried gel to Kodak XAR-5 film, pooled, and concentrated in a Speedvac (Savant Instruments, Inc.) to a volume of 50 μl. An equal volume of 2× SDS-PAGE sample buffer was added, and the samples were boiled for 2 to 5 min. Before being loaded on an SDS-PAGE (12% acrylamide) gel, the sample was centrifuged for 15 min at 100,000 × *g* to pellet particulate debris. The sample was electrophoresed with ^{125}I-labeled sample as a marker in a parallel lane.

The sample was then transferred onto an Immobilon-N membrane (Millipore Corp.) (a kind gift of Paul Matsudaira) at a constant current of 300 mA for 60 min, in a Bio-Rad Trans-Blot Apparatus with using 10 mM CAPS [3-(cyclohexylamino)-1-propanesulfonic acid] buffer containing 10% methanol (pH 11.0) as the transfer buffer. With the ^{125}I-labeled sample marker as a guide, a piece of membrane containing the p25 was carefully excised and used for sequencing.

Sequence analysis was performed on an Applied Biosystems 470A protein sequencer equipped with on-line phenylthiohydantoin analysis by using the RUN 470 program of Paul Matsudaira at the Massachusetts Institute of Technology Microsequencing Laboratory.

Phosphoamino acid analysis. Samples for phosphoamino acid analysis were prepared by a modified version of the technique described by Weiner et al. (29) and analyzed as described by Hunter and Sefton (12). The details have been described previously (20).

RESULTS

Turnover of p63/LMP. The high turnover rate of p63/LMP was demonstrated previously by means of [^{35}S]methionine pulse-chase analysis on cells treated with SDS, which solubilizes p63/LMP whether it is free or complexed in a form resistant to nonionic detergents (2, 20). Such experiments were performed with antibodies which recognize the carboxy-terminal half of the protein.

Since the carboxyl half of p63/LMP contains only one methionine residue, it would be impossible to detect specific degradation or cleavage products derived from this region by using [^{35}S]methionine. We therefore exploited the observation that p63/LMP is rich in proline residues, particularly at the carboxyl end, as an alternate approach to identifying cleavage or degradation products in a pulse-chase analysis. The analysis was performed with the S12 monoclonal antibody on cells pulsed with [^{3}H]proline and solubilized in SDS (see Materials and Methods). The results are shown in Fig. 1. We detected what appeared to be a specific cleavage product, of approximately 25,000 in molecular weight (hereafter referred to as p25), concomitant with the disappearance of p63/LMP. Interestingly, incorporation of proline, unlike that of methionine, was not maximal at the end of the pulse period. This is presumably due either to the larger size of the proline pool or to a lower rate of equilibration by proline. No other polypeptides were seen that were specifically precipitated. Therefore, this experiment suggests that p63/LMP is being specifically cleaved to produce the p25 polypeptide.

p25 is EBV specific and is detected in the cytoplasmic fraction. To confirm the EBV specificity of the p25 subfragment observed in the [^{3}H]proline pulse-chase analysis, we performed immunoprecipitations with the S12 antibody on membrane and cytoplasmic fractions from the EBV$^+$ BL line Jijoye and the EBV$^-$ BL line RAMOS labeled with [^{3}H]proline. The results are shown in Fig. 2. As expected, p63/LMP was clearly detected in the membrane fraction but not in the cytosolic fractions from Jijoye cells. Conversely, p25 was detected only in the cytoplasmic fraction. Neither p63/LMP nor p25 was precipitated with an isotype-matched antibody, nor were they detected in fractions from the EBV$^-$ BL line RAMOS. To further establish the specificity of the p25, we performed immunoprecipitations on the cytosolic fractions of two more EBV-positive (ER and RAJI) and two EBV-negative (BL-2 and K562) cell lines. The results are summarized in Table 1. p25 was detected in all of the EBV-infected cells and none of the uninfected cells. Lastly, to confirm the lack of methionine residues, we performed a parallel analysis on the ER line labeled with [^{35}S]methionine. p63 was readily detected in the membrane fractions, but p25 was undetected in the cytoplasmic fraction, even after a long exposure of the film (results not shown). Thus, p25 is rich in proline, poor in methionine, EBV specific, and localized in the cytoplasmic fraction.

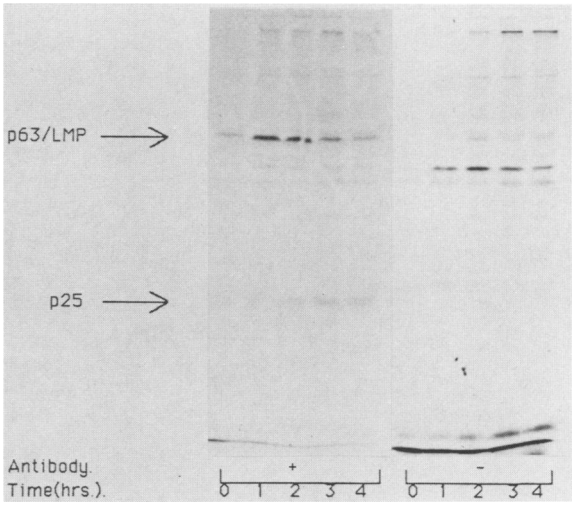

FIG. 1. Pulse-chase analysis of p63/LMP with [^{3}H]proline. Jijoye cells were pulsed with [^{3}H]proline for 20 min and harvested after being chased with nonradioactive medium for 0, 1, 2, 3, or 4 h. Immunoprecipitation was performed with the S12 monoclonal antibody (+) or an isotype-matched negative control (−). Samples were analyzed by SDS-PAGE (12% acrylamide) and fluorography. The exposure time for this autoradiogram was 4 months.

p25 is a phosphoprotein. It has been shown previously that p63/LMP was phosphorylated primarily on serine residues and weakly on threonine residues, but not on tyrosine (2, 20). To further explore the possibility that p25 was derived from p63/LMP and to test whether p25 contained the phosphorylated domain, we exploited this knowledge to provide an alternate, less time-consuming approach to characterizing p25.

When the membrane fractions of ^{32}P-labeled cells were immunoprecipitated (Fig. 3), phosphorylated p63/LMP was readily detected in two EBV$^+$ LCLs, ER and RKM, and the EBV$^+$ BL line Jijoye. As expected, pp63/LMP was not seen in immunoprecipitates from cytosolic fractions. However, a major phosphorylated polypeptide corresponding to p25 (hereafter referred to as pp25) was again detected in the cytoplasmic fractions.

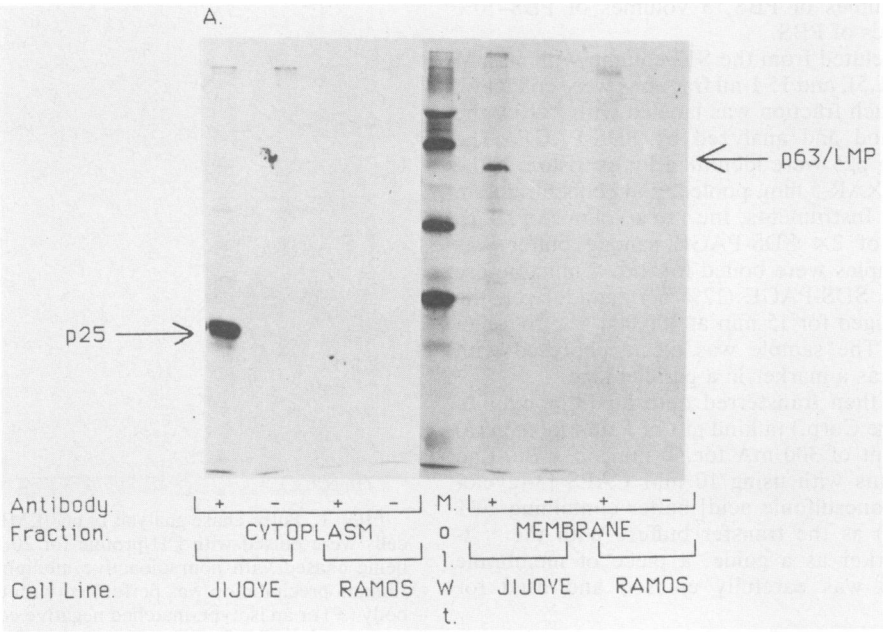

FIG. 2. Immunoprecipitation of p25 and p63/LMP labeled with [^{3}H]proline. Cells were labeled and lysed, and the cytoplasmic and membrane fractions were analyzed by immunoprecipitation with S12 (lanes +) or an isotype-matched negative control antibody (lanes −). Jijoye is an EBV$^+$ BL cell line; RAMOS is an EBV$^-$ BL cell line.

Neither pp63/LMP nor pp25 was detected in the EBV$^-$ control cell lines (BL-2 and RAMOS, EBV$^-$ BLs). In all, we have analyzed seven EBV$^+$ and five EBV$^-$ cell lines for pp25. We found that pp25 was detected only in the EBV$^+$ cells (Table 1).

Our previous analysis of p63/LMP revealed that it was phosphorylated at serine and threonine residues in a ratio of approximately 6:1 and that no phosphotyrosine was detected (20). Figure 4 shows phosphoamino acid analysis performed on pp25 and reveals essentially the same pattern of phosphorylation with a strong phosphoserine signal, a weak phosphothreonine signal, and no detectable phosphotyrosine (ratio 6:1:0). We conclude, therefore, that p25 is phosphorylated, consistent with its being derived from the phosphorylated domain of p63/LMP.

Expression of p63/LMP and p25 in transfected NIH 3T3

TABLE 1. Summary of cell lines tested for the expression of p25 in comparison with EBV status and expression of p63/LMP

Cell line	Origin	EBV status	p63/LMP expression	p25[a] expression
ER	EBV-LCL	+	+	+ (Pr, P_i)
JY	EBV-LCL	+	+	+ (P_i)
RKM	EBV-LCL	+	+	+ (P_i)
Raji	BL	+	+	+ (Pr, P_i)
AG876	BL	+	+	+ (P_i)
Jijoye	BL	+	+	+ (Pr, P_i)
BL-36	BL	+	+	+ (P_i)
BL-1	BL	−	−	− (P_i)
BL-2	BL	−	−	− (Pr, P_i)
BL-31	BL	−	−	− (P_i)
RAMOS	BL	−	−	− (Pr, P_i)
K562	Erythroleukemia	−	−	− (Pr, P_i)
NIH 3T3	Murine fibroblast	−	−	− (P_i)
NIH 3T3 + pSV2gpt		−	−	− (P_i)
NIH 3T3 + pSV2gpt LMP		−	+	+ (P_i)
BALB/c 3T3	Murine fibroblast	−	−	− (P_i)
BALB/c 3T3 + pSV2neo		−	−	− (P_i)
BALB/c 3T3 + pSV2BNLFI		−	+	+ (P_i)
Rat-1	Rat fibroblast	−	−	− (P_i)
Rat-1 + pSV2neo/pSV2gpt		−	−	− (Pr, P_i)
Rat-1 + pSV2BNLFI/pSVgptLMP		−	+	+ (Pr, P_i)
DG75	BL	−	−	− (P_i)
DG75-LMP		−	+	+ (P_i)

[a] p25 was detected after labeling with [^{3}H]proline (Pr) or $^{32}P_i$ (P_i).

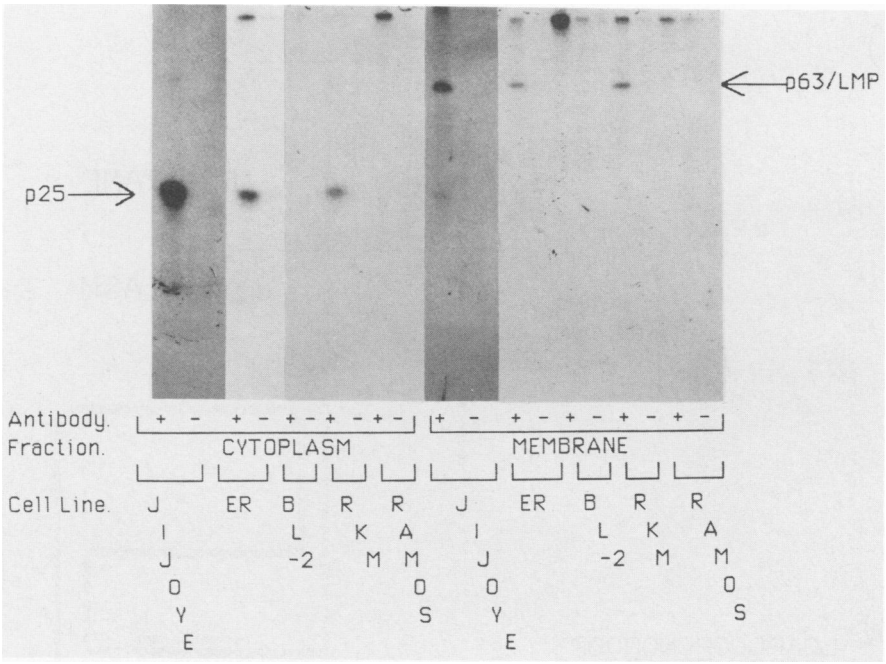

FIG. 3. Immunoprecipitation of p25 and p63/LMP labeled with $^{32}P_i$. For details, see the legend to Fig. 2. ER and RKM are EBV$^+$ LCLs; Jijoye is an EBV$^+$ BL cell line; BL-2 and RAMOS are EBV$^-$ BL cell lines.

cells. The *Bam*HI NJ fragment of the EBV genome that encodes p63/LMP does not contain an open reading frame for a 25-kilodalton protein, and no latent RNA transcript to encode such a protein has been mapped to this region. Thus, the detection of pp25 in rodent cells transfected with this fragment would provide compelling evidence that pp25 was derived by posttranslational processing of p63/LMP.

NIH 3T3 cells were transfected with the pSV2gpt-MTLM vector, and stable colonies were selected, grown up, labeled with ^{32}P, and subjected to immunoprecipitation analysis with S12 antibody or an isotype-matched control. [^{32}P]pp63/LMP and [^{32}P]pp25 were readily detected in the membrane fraction and cytosolic fractions, respectively, of two independently derived colonies (R3 and R5) (Fig. 5). Neither polypeptide was detected in untransfected NIH 3T3 cells or in cells from a colony transfected with the pSV2gpt plasmid alone. The correct sizes of pp63/LMP and pp25 were confirmed by electrophoresis in parallel with the phosphoproteins derived from the Jijoye cell line (results not shown). Furthermore, no fragments of intermediary size between

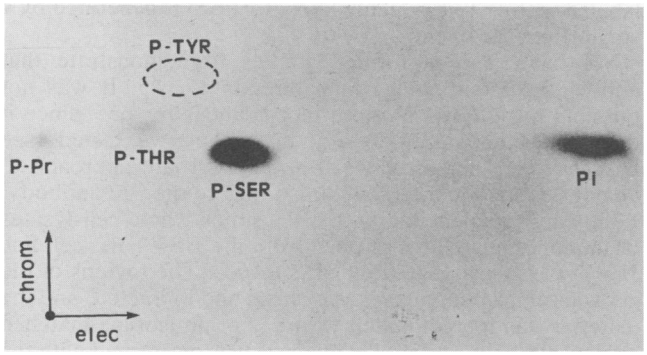

FIG. 4. Phosphoamino acid analysis of pp25. Radiolabeled, immunoprecipitated pp25 was isolated and extracted from an SDS-polyacrylamide gel for hydrolysis as described in Materials and Methods. Mixtures of radioactive and nonradioactive carrier phosphoamino acids were chromatographed. The nonradioactive carriers were located by ninhydrin staining, and the radioactive amino acids were located by fluorography. Abbreviations: P-Pr, incompletely hydrolyzed protein; P_i, free phosphate; P-THR, P-SER, P-TYR, phosphotreonine, phosphoserine, and phosphotyrosine, respectively.

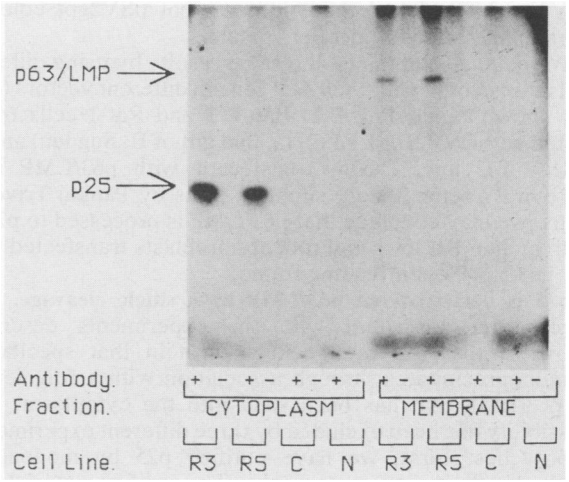

FIG. 5. pp25 is expressed in NIH 3T3 cells transfected with the p63/LMP gene. NIH 3T3 cells were transfected with a plasmid containing the *Bam*HI NJ fragment of EBV that encodes p63/LMP (pSV2gpt LMP) or pSV2gpt alone. Independent colonies were picked, grown up, and analyzed as in Fig. 2, except that the radiolabel was $^{32}P_i$. Abbreviations: N, NIH 3T3 untransfected control; R3 and R5, two independent clones of NIH 3T3 cells transfected with pSV2gpt LMP; C, NIH 3T3 transfected with pSV2gpt.

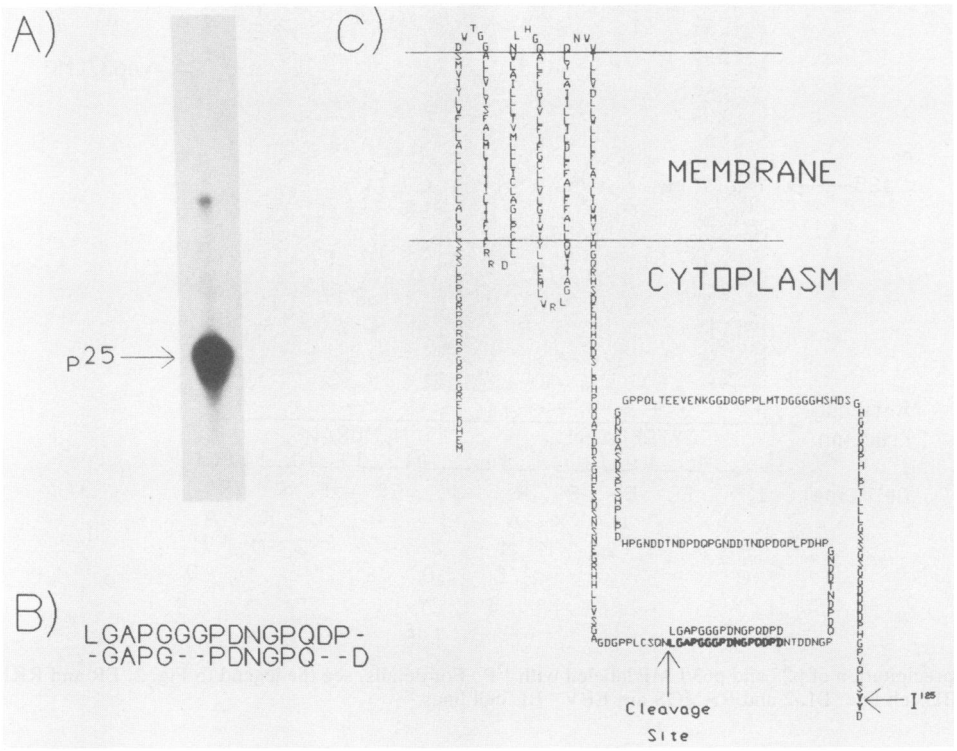

FIG. 6. Identification of the cleavage site for the generation of p25. (A) p25 was purified from the cytoplasm of Jijoye cells by antibody affinity chromatography with the S12 antibody. The purified material was radiolabeled with ^{125}I by the chloramine-T method and analyzed by SDS-PAGE. The minor contaminating larger polypeptide has a molecular weight consistent with the heavy chain from the S12 immunoglobulin column. The major p25 polypeptide was eluted for sequencing. (B) Amino-terminal amino acid sequence of purified p25. The two sequences shown represent parallel sequences derived from the same purified preparation. (C) Diagrammatic representation of the sequence of p63/LMP derived from the B95-8 strain (1) cell membrane. The homologous sequence to that derived in panel B is shown in boldface and labeled "Cleavage site." Also shown in boldface and labeled "^{125}I" are the only two tyrosine residues in the p25 sequence available for iodination by chloramine-T.

pp63/LMP and pp25 were detected. In all, five independent pSV2gpt-MTLM and three independent pSV2gpt colonies were analyzed, with identical results.

We have also obtained the same results by using different cells transfected with the LMP gene in different vectors (data not shown), including BALB/c 3T3 and Rat-1 cells transfected with pSV2BNLF1 (2) (a kind gift of B. Sugden) and an EBV^- BL line, DG75, transfected with p63/LMP in a retroviral vector (kindly supplied to us by Pankaj Trivedi). Thus, we may conclude that p63/LMP is processed to p25 in both human BL lines and rodent fibroblasts transfected with the p63/LMP open reading frame.

p25 is derived from p63/LMP by a single cleavage. One explanation consistent with the experiments described above is that p25 is a cellular protein that specifically immunoprecipitates through association with a fragment(s) of p63/LMP that has been shed into the cytoplasm. This possibility has been excluded by three different experimental approaches. First, we have purified p25 by monoclonal antibody affinity chromatography. The purified material consisted of a major single polypeptide at 25 kilodaltons and a minor component, consistent in size with being contaminating immunoglobulin heavy chain from the antibody column (Fig. 6A). The 25-kilodalton polypeptide was excised and subjected to amino acid sequencing. Two sequences were obtained, which generated a 16-amino-acid continuous sequence (Fig. 6B) that was 100% identical to a region in the middle of the cytoplasmic tail of the published p63/LMP sequence from the B95-8 strain (5, 11) (Fig. 6C). Identical results were obtained with p25 purified from the ER LCL (not shown). Interestingly, since the only residues that may be iodinated by the chloramine-T method are the second and third most carboxy-terminal tyrosines and p25 is readily labeled with ^{125}I, it is highly likely that p25 is generated by a single cleavage event.

We have also performed studies to demonstrate that anti-p63/LMP antibodies bind directly to p25. It was not possible to perform Western blot analysis for p25, since it does not bind stably to any membrane we tested (see Discussion). Therefore, we took an alternate approach to demonstrating the direct binding of p25 to the S12 antibody. Following radiolabeling with $^{32}P_i$, either whole-cell lysates or immunoprecipitates of pp25 from the EBV^+ BL cell line Jijoye were fractionated by SDS-PAGE. The regions of the gel where pp25 migrates was excised and extracted, and the material was reprecipitated with S12 or an isotype-matched control antibody. A parallel analysis was performed with the EBV^- BL line BL-2. The data for the whole-cell lysates are given in Fig. 7, which shows that pp25 was readily reprecipitated with S12 from the 25-kilodalton region of the gel containing only the Jijoye extracts but not the BL-2 extracts. Identical results were obtained with the EBV^+ LCL ER and the EBV^- line K562 (data not shown). Thus, S12 binds directly to pp25.

Immunoprecipitation experiments were also carried out (data not shown) with a panel of three monoclonal antibodies

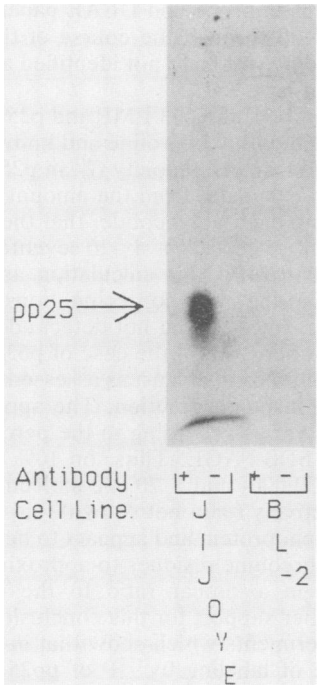

FIG. 7. pp25 binds directly to the S12 antibody. Cells prelabeled with $^{32}P_i$ were lysed directly into sample buffer and subjected to SDS-PAGE. A gel slice containing the region where pp25 migrates was removed, and the labeled polypeptides were eluted and precipitated with the S12 anti-p63/LMP (+) or control (−) antibodies. Jijoye is an EBV$^+$ BL cell line; BL-2 is an EBV$^-$ BL cell line.

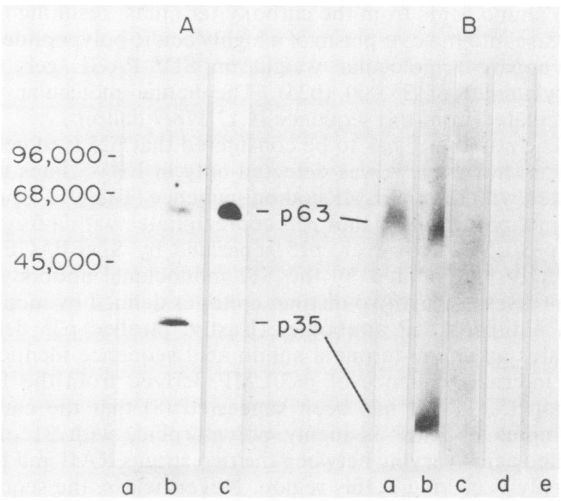

FIG. 8. Detection of a p35 amino-terminal fragment of p63 in plasma membrane. (A) Plasma membranes were isolated from the EBV$^+$ BL Jijoye line and tested by Western blot analysis for reactivity with affinity-purified rabbit anti-N serum (lane b) or the S12 monoclonal antibody (lane c). Molecular weight standards are shown in lane a. (B) As in panel A, except that the lanes were probed with S12 monoclonal antibody (lane a), affinity-purified rabbit anti-N serum preincubated with a control peptide (lane b) or with peptide N (lane c), negative control rabbit antiserum (flowthrough from the peptide affinity column) incubated as in lane b (lane d), and negative control rabbit antiserum preincubated with peptide N and incubated as in lane b (lane e).

raised against the carboxy-terminal half of p63/LMP, which recognizes two distinct epitopes, one defined by CS-2 and one defined by CS-3 and CS-4. In addition, immunoprecipitation was performed with the rabbit anti-N serum and S12. The panel of three monoclonal antibodies all immunoprecipitated pp25. Furthermore, the rabbit anti-N serum did not precipitate pp25, although in a parallel analysis it did precipitate p63/LMP. Thus, pp25 bears two distinct epitopes in common with the carboxyl terminus of pp63/LMP, but lacks the amino-terminal sequence.

Identification of a membrane-associated subfragment of p63/LMP. If the short half-life of p63/LMP were due to cleavage and release of the phosphorylated cytoplasmic end, it would be predicted that an amino-terminal peptide may be detected in the membrane fraction. No such peptide was seen in [^{35}S]methionine pulse-chase analysis performed with either the S12 antibody or a rabbit serum directed to the amino terminus of p63/LMP.

Both analyses reveal identical results, namely, the disappearance of pulse-labeled p63/LMP with a half-life of about 2 h (data not shown). As described previously (20) and above, no processed products were detected in the membrane or cytosol fractions. A ladder of smaller and fainter polypeptides was also precipitated, as described previously (2). However, the label in these polypeptides decreased at the same rate as intact p63/LMP. There is no evidence that these forms are derived by processing of p63/LMP, since the label in the intact protein does not chase into the smaller proteins.

Since the putative membrane-associated fragment of p63/LMP would consist predominantly of hydrophobic residues, it could remain undetected in immunoprecipitation analysis as a result of being insoluble in the detergent buffers. We have therefore attempted to use an alternate approach to detect an amino-terminal fragment of p63/LMP by performing Western blots on the membrane fraction with the rabbit anti-N antiserum. The rabbit anti-N antiserum did react with both the intact p63/LMP and an additional 35,000-molecular-weight polypeptide (p35) (Fig. 8A, lane b). The specificity of this reactivity was confirmed in two ways. First, as expected, S12 did not react with the p35 (Fig. 8A, lane c); second, reactivity with the p35 was specifically inhibited by preincubation of the antiserum with the immunizing peptide (Fig. 8B, lane c), but was not inhibited with a random irrelevant peptide (Fig. 8B, lane b). Lastly, p35 was not detected in membrane fractions from the EBV$^-$ BL line RAMOS (data not shown).

We performed an experiment to test the possibility that p35 was not detected by immunoprecipitation analyses as a result of insolubility in the detergent buffers. A membrane fraction from Jijoye cells was directly solubilized in the detergent buffers used in immunoprecipitation analysis. This material was then separated into soluble and insoluble fractions, which were subjected to Western blot analysis by using the rabbit anti-N antiserum. p35 was detected only in the insoluble fractions (data not shown).

DISCUSSION

It has been shown previously that p63/LMP becomes associated with the cytoskeleton, with a very short half-life (30 min) (20). This complexed form is resistant to nonionic detergents, but may be solubilized by 8 M urea or boiling in SDS. Thereafter, it turns over with a short half-life (about 2 h) (2, 20). In the work described in this paper we show that the turnover is due to a specific single cleavage of p63/LMP

145 amino acids from the carboxy terminus, resulting in the release into the cytoplasm of a highly acidic polypeptide with an apparent molecular weight on SDS-PAGE gels (12% acrylamide) of 25,000 (p25). The actual molecular mass calculated from the sequence is 17,178.7 daltons.

The possibility has to be considered that p25 is of cellular origin; however, it was detected only in EBV$^+$ lines transfected with the p63/LMP coding sequence (Table 1). Another formal possibility is that p25 coprecipitates with a fragment of p63/LMP. However, p25 separated by SDS-PAGE was directly precipitated by the S12 monoclonal antibody and expresses at least two distinct epitopes defined by monoclonal anti-p63/LMP antibodies. Lastly, purified p25 demonstrates an amino-terminal amino acid sequence identical to an internal sequence of p63/LMP derived from the B95-8 strain (5, 11). It has been suggested (8) that the carboxy terminus of LMP is highly polymorphic, with 21 of 386 amino acids varying between the two strains RAJI and B95-8 mostly occurring in this region. Nevertheless, the sequence 8 amino acids either side of the cleavage site is identical in RAJI, B95-8, and Jijoye cells. This is despite the fact that B95-8 and Jijoye are biologically distinct strains of A and B type, respectively.

Several pieces of evidence support the conclusion that p25 is derived by specific proteolytic processing of p63/LMP. First, the pulse-chase analysis reveals that p25 is the only other detectable product that was specifically immunoprecipitated and also increased concomitant with the decrease in p63/LMP. Quantitation of p63/LMP and p25 by scanning the pulse-chase film reveals that p63/LMP is disappearing, as shown previously, with a half-life of about 2 h. p25, however, is produced more slowly, not consistent with the simple half-life disappearance rate of p63. Rather, the kinetics are consistent with the conclusion that p25 appears with a half-life of 1.5 h and then turns over with a half-life of about 6 h. Second, p25 was readily detected when immunoprecipitation was performed on whole-cell lysates that were directly solubilized by boiling in SDS (Fig. 7), which should minimize nonspecific proteolysis. Third, p25 was never detected in immunoprecipitates from purified membrane preparations, in which proteolysis could potentially occur. Lastly, the EBV *Bam*HI NJ fragment used to transfect the NIH 3T3 cells does not have an open reading frame for a 25,000-molecular-weight protein. Nevertheless, p25 and no other fragments was readily detected in the transfected cells.

It has been suggested previously (2) that p63/LMP is degraded through a stepladder of progressively smaller molecules. This conclusion is contradictory to our own, and we believe that it is unlikely to be correct, since it would predict that, in pulse-chase experiments, radioactivity should increase in smaller polypeptides as it decreases in p63/LMP. Such a result was not seen in our own experiments or in the studies of Baichwal and Sugden (2). Rather, the intensity of label in the smaller bands decreased in parallel with that in p63/LMP. Thus, the stepladder effect is probably generated by proteolysis of p63/LMP during the experiment.

p25 was not detected previously by Western blot analysis of cytoplasmic fractions, although Hatzubai et al. (9), who used a radioimmunoassay, have reported antigenic activity related to p63/LMP. We have observed (data not shown) that p25 does not bind to nitrocellulose or polyvinylidene difluoride membranes. Thus, failure to detect p25 in Western blots was due to the absence of p25 from the matrix. Failure to bind could occur because p25 contains a large excess of acidic over basic residues, is phosphorylated, and has a pI of <3.5. p25 does bind efficiently to positively charged membranes such as Zeta probe and DEAE paper; however, it is readily washed off through the course of the Western blot procedure. To date, we have not identified a suitable matrix for p25 Western blot.

Assuming that both the p63/LMP and p25 molecules were uniformly labeled with [^{3}H]proline and knowing the number of proline residues in each moiety (24 in p25 and 36 in p63), it is possible to calculate, from the amount of radioactivity incorporated into each polypeptide, that there is about 2 ng of p25 per 10^6 Jijoye cells, or a six- to sevenfold molar excess of p25 over p63/LMP. This calculation assumes close to 100% recovery in the immunoprecipitations, which is probably reasonable, since we did not detect additional material upon reprecipitation and, in the case of p63/LMP, all of the material was solubilized, at least as assessed by Western blot analysis of the insoluble fraction. The apparent molecular weight of pp25 varies according to the percentage of acrylamide used in SDS-PAGE. Thus, on 10% and 18% gels it migrates as a 30,000- and a 20,000-molecular-weight polypeptide, respectively (data not shown).

p25 is a phosphoprotein and appears to be phosphorylated on serine and threonine residues to approximately the same extent as and in a similar ratio to the intact p63/LMP molecule. Further support for this conclusion comes from a number of experiments which show that in Jijoye cells, the relative degree of labeling by ^{32}P of pp25 compared with pp63 varied between 5:1 and 8:1. This is very close to the molar ratio of protein present (see above), suggesting that the extent of phosphorylation of pp63/LMP is similar to that for p25.

p25 possesses a high proportion of serine and threonine residues. Nevertheless, experiments in progress suggest that the site of phosphorylation is located primarily among the three serine residues around position 311 (Moorthy and Thorley-Lawson, unpublished data). We have previously shown that all of the phosphorylated p63/LMP is in the fraction associated with the cytoskeleton, i.e., resistant to nonionic detergents and present in the plasma membrane (20). Thus, the simplest order of events would be that p63/LMP migrates to the plasma membrane, where it becomes associated with the cytoskeleton with a half-life of about 30 min. The protein is then phosphorylated and cleaved with a half-life of 1.5 to 2 h. It is tempting to speculate that phosphorylation itself could be the signal for cleavage.

The release of p25 into the cytoplasm would leave behind an amino-terminal fragment that may be represented by the p35 we have detected. In the absence of pulse-chase analysis, it is not possible to conclude that p35 is derived from p63/LMP. Nevertheless, the presence of amino-terminal epitopes, the absence of carboxy-terminal epitopes, and the molecular weight and membrane location of p35 are consistent with this conclusion. Since the amino-terminal half of p63/LMP is sufficient to transform rodent cells (3), p35 should also perform this function. It is unclear at present what role p25 plays in transformation. For example, its role may be more critical in the normal human B cell than in the immortalized rodent fibroblast used in the transformation studies. Alternatively, cleavage may actually be required to generate the functional form of p63/LMP.

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LITERATURE CITED

1. **Aman, P., B. Ehlin-Henriksson, and G. Klein.** 1984. Epstein-Barr virus susceptibility of normal human B lymphocyte populations. J. Exp. Med. **159**:208–213.
2. **Baichwal, V. R., and B. Sugden.** 1987. Posttranslational processing of an Epstein-Barr virus-encoded membrane protein expressed in cells transformed by Epstein-Barr virus. J. Virol. **61**:866–875.
3. **Baichwal, V. R., and B. Sugden.** 1988. Transformation of Balb 3T3 cells by the BNLF-1 gene of Epstein-Barr virus. Oncogene **2**:461–468.
4. **Bradford, M. M.** 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
5. **Fennewald, S., V. van Santen, and E. Kieff.** 1984. Nucleotide sequence of a mRNA transcribed in latent growth-transforming infection indicates that it may encode a membrane protein. J. Virol. **51**:411–419.
6. **Gordon, J., L. Walker, G. Guy, G. Brown, M. Rowe, and A. Rickinson.** 1986. Control of human B-lymphocyte replication. II. Transforming Epstein-Barr virus exploits three distinct viral signals to undermine three separate control points in B-cell growth. Immunology **58**:591–595.
7. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay for infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
8. **Hatfull, G., A. T. Bankier, B. G. Barrell, and P. J. Farrell.** 1988. Sequence analysis of Raji Epstein-Barr virus DNA. Virology **164**:334–340.
9. **Hatzubai, A., M. Anafi, M. G. Masucci, J. Dillner, R. A. Lerner, G. Klein, and D. Solitzeanu.** 1987. Down-regulation of the EBV-encoded latent membrane protein (LMP) in Burkitt lymphoma lines. Int. J. Cancer **40**:358–364.
10. **Hennessy, K., S. Fennewald, M. Hummel, T. Cole, and E. Kieff.** 1984. A membrane protein encoded by Epstein-Barr virus in latent growth-transforming infection. Proc. Natl. Acad. Sci. USA **81**:7207–7211.
11. **Hudson, G. S., P. J. Farrell, and B. G. Barrell.** 1985. Two related but differentially expressed potential membrane proteins encoded by the *Eco*RI Dhet region of Epstein-Barr virus B95-8. J. Virol. **53**:528–535.
12. **Hunter, T., and B. M. Sefton.** 1980. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. USA **77**:1311–1315.
13. **Hutt-Fletcher, L. M.** 1987. Synergistic activation of cells by Epstein-Barr virus and B-cell growth factor. J. Virol. **61**: 774–781.
14. **Kallin, B., J. Dillner, I. Ernberg, B. Ehlin-Henriksson, A. Rosen, W. Henle, G. Henle, and G. Klein.** 1986. Four virally determined nuclear antigens are expressed in Epstein-Barr virus-transformed cells. Proc. Natl. Acad. Sci. USA **83**:1499–1503.
15. **Kessler, S. W.** 1975. Rapid isolation of antigens from cells with a staphylococcal protein-A antibody absorbent. J. Immunol. **115**:1617–1624.
16. **Liebowitz, D., and E. Kieff.** 1986. Orientation and patching of the latent infection membrane protein encoded by Epstein-Barr virus. J. Virol. **58**:233–237.
17. **Liebowitz, D., R. Kopan, E. Fuchs, J. Sample, and E. Kieff.** 1987. An Epstein-Barr virus transforming protein associates with Vimentin in lymphocytes. Mol. Cell. Biol. **7**:2299–2308.
18. **Liu, F. T., M. Zinnecker, T. Hamaoka, and D. H. Katz.** 1979. New procedures for preparation and isolation of conjugates of proteins and a synthetic copolymer of D-amino-acids and immunochemical characterization of such conjugates. Biochemistry **18**:690–697.
19. **Mann, K. P., D. Staunton, and D. A. Thorley-Lawson.** 1985. An Epstein-Barr virus-encoded protein found in the plasma membranes of transformed cells. J. Virol. **55**:710–720.
20. **Mann, K. P., and D. Thorley-Lawson.** 1987. Posttranslational processing of the Epstein-Barr virus-encoded p63/LMP protein. J. Virol. **61**:2100–2108.
21. **Miller, G., J. F. Enders, and H. I. Lisco.** 1969. Establishment of lines from normal human blood leukocytes by cocultivation with a leukocyte line derived from a leukemic child. Proc. Soc. Exp. Biol. Med. **132**:247–252.
22. **Mulligan, R. C., and P. Berg.** 1981. Selection of animal cells that express the E. coli gene coding for xanthine-guanine phosphoribosyltransferase. Proc. Natl. Acad. Sci. USA **78**:2072–2076.
23. **Rowe, M., H. S. Evans, L. S. Young, K. Hennessy, E. Kieff, and A. B. Rickinson.** 1987. Monoclonal antibodies to the latent membrane protein of Epstein-Barr virus reveal heterogeneity of the protein and inducible expression in virus-transformed cells. J. Gen. Virol. **68**:1575–1586.
24. **Thorley-Lawson, D. A., and K. Geilinger.** 1980. Monoclonal antibodies against the major glycoprotein (gp350/220) of Epstein-Barr virus neutralize infectivity. Proc. Natl. Acad. Sci. USA **77**:5307–5311.
25. **Thorley-Lawson, D. A., and K. P. Mann.** 1985. The early effects in EBV infection provide a model for B cell activation. J. Exp. Med. **162**:45–49.
26. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
27. **Wang, D., D. Liebowitz, and E. Kieff.** 1985. An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells. Cell **43**:831–840.
28. **Wang, D., D. Liebowitz, F. Wang, C. Gregory, A. Rickinson, R. Larson, T. Springer, and E. Kieff.** 1988. Epstein-Barr virus latent infection membrane protein alters the human B-lymphocyte phenotype: deletion of the amino terminus abolishes activity. J. Virol. **62**:4173–4184.
29. **Weiner, A. M., T. Platt, and K. Weber.** 1972. Amino-terminal sequence analysis of proteins purified on a nanomole scale by gel electrophoresis. J. Biol. Chem. **247**:3242–3251.