Epstein-Barr Virus-Encoded Protein Found in Plasma Membranes of Transformed Cells

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We have developed monoclonal antibodies to a 63,000-molecular-weight protein (p63) which is the product of the most abundant messenger RNA in Epstein-Barr virus-transformed cells and shown that the protein is associated specifically with plasma membranes. It was also found to be associated with the other membrane fractions and was found in all Epstein-Barr virus-transformed cells tested. In addition, p63 was present in virions, resulting in transient, early appearance in newly infected cells. Newly synthesized p63 was detected at the time cells underwent blast transformation (48 to 72 h postinfection). The possible role of this protein in transformation and as a target for cell-mediated cytotoxicity is discussed.

Epstein-Barr virus (EBV) is the causative agent of infectious mononucleosis (19), a self-limiting lymphoproliferative disease. The viral infection persists for the life of the host without subsequent clinical symptoms, and maintenance of this subclinical state is presumably due to the presence of virus-specific neutralizing antibody and cytotoxic T cells. EBV is also associated with human malignancies including African Burkitt's lymphoma (9, 19, 29), nasopharyngeal carcinoma (21, 33), and polyclonal lymphomas in immunosuppressed individuals (2, 5). EBV has a unique tropism for resting B lymphocytes (30, 37, 44, 48), and in vitro, it causes these cells to transform into continuously proliferating lymphoblasts (16, 20, 34). This process has been termed growth transformation or immortalization. Three major mRNAs have been observed in lymphoblastoid cell lines (25, 35, 45); one of these mRNAs is already known to encode the Epstein-Barr nuclear antigen (14, 23). The most abundant mRNA is a product of a gene present in the *EcoRI*-Dhet fragment of the EBV genome (1, 13). The nucleotide-coding sequence of this gene has been determined in two laboratories (1, 13), and the protein structure has been predicted (13, 24). It has also been predicted (13) that the protein would have a molecular weight of 42,000, a highly charged amino terminus followed by a large hydrophobic domain with three reverse turn loops separated by alpha helices, and a large hydrophilic carboxy terminus with no predicted secondary structure. The hydrophobic domains are typical of structures found in membrane-bound ion channels, and, therefore, this protein, if expressed, is probably a membrane protein that could be involved in changes in ion permeability in the transformed cell.

Prior evidence for the presence of an EBV-associated plasma membrane protein in transformed cells comes from studies of cell-mediated immunity (30, 38, 40, 46). These studies have shown that it is possible to generate cytotoxic T-cell clones that recognize a target, termed lymphocyte-determined membrane antigen, restricted to EBV-infected cells. The biochemical nature of this antigen remains unclear. An EBV-encoded membrane protein could also play a role in growth transformation analogous to that of plasma membrane-associated products of known oncogenes (for a review, see reference 28).

The carboxy half of the protein encoded by the *EcoRI*-Dhet fragment has been expressed in *Escherichia coli* as a fusion protein with beta-galactosidase (22). In this report, we describe the production of monoclonal antibodies that recognize the EBV-encoded portion of this fusion protein and show that they identify a 63,000-molecular-weight protein present both on the plasma membranes of EBV-transformed cells and in the Epstein-Barr virion. While this manuscript was in preparation, Hennessy et al. (22) described a rabbit antiserum against the same fusion protein that reacted with a polypeptide of similar size present in the membrane-microsomal fraction of EBV-transformed cells.

MATERIALS AND METHODS

Production of monoclonal antibodies. The fusion protein (pKH548) (kind gift of K. Hennessy and E. Kieff), consisting of 49% of the protein (189 amino acids from the carboxy terminus) coupled to beta-galactosidase, was made and isolated as described previously (23). Its characterization has been described elsewhere (22). pKH548 (50 µg) was suspended in 50 mM Tris buffer (pH 7.5)–100 mM 2-mercaptoethanol, emulsified in complete Freund adjuvant (the kind gift of S. Leskowitz), and injected either intraperitoneally or in the footpads and the base of the tail of BALB/c female mice. At least 1 month later, each mouse was given an intravenous boost of 50 µg of fusion protein in saline. Splenocytes were prepared 3 days later and fused with cells from the P3X63Ag8.653 myeloma line in a 2:1 ratio by the procedure of Kohler and Milstein (26) with modifications. Selective media contained 100 µM hypoxanthine-azaserine (1 µg/ml; Sigma Chemical Co.) (15). Fused cells were left in bulk culture overnight and then distributed among 10 96-well plates (Co-Star; Data Packaging Corp.). We obtained 250 hybridomas. The hybridoma supernatants were screened for binding to beta-galactosidase (Sigma) and to pKH548 by using an enzyme-linked immunosorbent assay. Briefly, 96-well plates (Dynatech Laboratories, Inc.) were coated with beta-galactosidase or pKH548 at a concentration of 1 mg/100 µl of 20 mM borate buffer (pH 8.6)–1.4 M NaCl and were incubated for 2 h at 37°C. The plates were washed three times with phosphate-buffered saline (PBS) containing 1% Tween 20 (Sigma) and three times with PBS alone. Five percent bovine serum albumin (BSA) in PBS (PBS–5% BSA) was added to each well to block nonspecific binding sites. The

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FIG. 1. Identification of p63 by immunoblotting. Total membranes from the cell lines RAMOS (lanes A and D), JY (lanes B and E), and RAJI (lanes C and F) were screened with the S12 monoclonal antibody (lanes A through C) and an isotype-matched negative control antibody (lanes D through F).

plates were incubated at 20°C for 2 h and washed as above. Undiluted culture supernatants were added to each well, and the plates were incubated and washed as before. Goat anti-mouse immunoglobulin G(IgG) conjugated to alkaline phosphatase (Sigma) in 0.1% Tween 20 was added to each well, and the plates were incubated overnight and washed as above. The alkaline phosphate substrate disodium *p*-nitrophenyl phosphate (5 mg/ml; Sigma) in 50 mM sodium bicarbonate (pH 9.5)–2 mM magnesium chloride was added to each well. A MicroELISA Autoreader MR 580 (Dynatech) was used to screen the cells. Supernatant containing antibody from a hybridoma (IG12D9) specific for beta-galactosidase (the kind gift of M. Boris Rotman) was used as a positive control in the assay. We obtained 43 hybridomas specific for beta-galactosidase as well as 5 hybridomas specific for pKH548. Of these five, three were retained as stable hybridomas after two subclonings. Ascites fluid was produced, and isotypes and reactivity with protein A were determined. Isotyping was performed by Ouchterlony analysis with isotype-specific antisera (Gateway Immunosera Co.). Protein A reactivity was determined by retention on a Sepharose-protein A (Sigma) column.

Cytofluorographic analysis. Cells were prepared for analysis as follows. Cells (10^6 per sample) were pelleted at $300 \times g$ for 10 min in the HL-8 rotor of a Sorvall RC3 centrifuge and then suspended either in RPMI medium containing 1% fetal calf serum (FCS) (medium) or in PBS containing 1% Formalin and 5% sucrose for fixation. After 20 min of

incubation, the fixed cells were washed three times in medium. After the final wash, both fixed and unfixed cells were suspended and incubated for 20 min in 100 μ l of antibody either as a 1:1 dilution of supernatant or as a 1:100 dilution of ascites in PBS. The cells were then washed three times, suspended in 100 μ l of fluorescein isothiocyanate–goat anti-mouse IgG conjugate (Cappel Laboratories) at a 1:20 dilution, and incubated for a further 20 min. The cells were again washed three times and after the final pelleting were suspended in 1 ml of medium. All steps were performed at 4°C, and all antibodies were centrifuged for 20 min at $100,000 \times g$ in a Beckman airfuge before use. The cells were analyzed on a fluorescence-activated cell sorter (model IV; Becton Dickinson and Co.).

Immunofluorescence. Samples for immunofluorescence were prepared by centrifuging 2×10^5 cells in 200 to 400 μ l onto slides with a Shandon Southern Cytospin. The smears were allowed to dry and then were fixed in methanol at –20°C for 3 to 4 min. Excess methanol was then shaken off, and the slides were air dried. The first antibody was added (same dilution as previously), and the slides were incubated for 30 min at room temperature. Slides were washed with PBS and incubated for 30 min with fluorescein isothiocyanate–goat anti-mouse IgG (described above). The slides were again washed extensively with PBS.

To detect and photograph low-level fluorescence, a Venus three-stage low-light-level television camera on a Zeiss inverted fluorescence microscope was used for image enhancement where noted.

Immunoblots. Total membrane preparations were used except where stated. These samples were prepared by pelleting 10^7 cells and suspending them in cold lysing buffer (10 mM Tris [pH 7.5], 1 mM KCl, 1 mM $MgCl_2$). This solution was freeze-thawed twice. The nuclei were pelleted by spinning at $800 \times g$ in the HL-8 rotor of Sorvall RC3 centrifuge for 15 min. These pellets were washed twice more, and all supernatants were pooled. The pooled supernatants were spun at $100,000 \times g$ for 1 h in the 50 Ti rotor of a Beckman L5-50B ultracentrifuge, and the total membrane pellet was suspended in 100 μ l of lysing buffer. Plasma membranes and other subcellular fractions were purified by the method of Crumpton and Snary (6) with modifications (11). Purified virions were prepared by the method of Dolyniuk et al. (10). Protein concentrations were estimated by the method of Bradford (3), using the Bio-Rad protein assay. Samples (25 to 50 μ g) were prepared for sodium dodecyl sulfate–polyacrylamide gel electrophoresis, which was performed on sodium dodecyl sulfate–10% polyacrylamide gels by the method of Laemmli (27). Immunoblots were performed by the method of DeBlas and Cherwinski (8) with modifications. Proteins were transferred from the gel to nitrocellulose membranes for 2 h at 300 mA or overnight at 30 mA. The gel was then stained with Coomassie blue to determine the efficiency of transfer. The nitrocellulose sheet was immersed in PBS–5% BSA and incubated for 2 h at 37°C. It was then transferred to fresh PBS–5% BSA containing a monoclonal antibody specific for pKH548 (preparation described above) and incubated for 2 h at room temperature. The nitrocellulose sheet was then washed once for 10 min in PBS, twice for 20 min each in PBS containing 0.05% Nonidet P-40 and 5% saturated ammonium sulfate, and once more for 10 min in PBS. The nitrocellulose sheet was immersed in fresh PBS–5% BSA with 2.5×10^5 to 5×10^5 cpm of iodinated protein A (specific activity, 8.3×10^8 cpm/ μ g per ml) per ml, incubated for 2 h at room temperature, and washed as above.

FIG. 2. Immunofluorescence on EBV-positive and -negative cell lines. Immunofluorescence was performed with the S12 monoclonal antibody upon the methanol-fixed cell lines BH (A) and RAMOS (B).

Transfection. Transfection of NIH 3T3 mouse fibroblasts was performed by the CaPO_4 precipitate method of Graham and Van der Ebb (17). The plasmid containing the gene encoding gentamicin (G418; GIBCO Laboratories) resistance, pSV2neo (the kind gift of Ted Krontiris), was transfected alone or was cotransfected with the plasmids containing

fused viral termini in MVA-3 (pCB281), *Bam*HI-L in pBR322 (pDK39) (the kind gift of T. Dambaugh [7]), or *Bam*HI-K in the cosmid MV8-A (the kind gift of G. Miller). Transfected cells were selected for by the addition of G418 (500 $\mu\text{g}/\text{ml}$) to the culture 48 h posttransfection. At day 8, colonies were pooled and cultured for an additional week before assaying

FIG. 3. Immunoblotting on subcellular fractions. (a) The following subcellular fractions of the cell line BH were tested for the presence of p63: plasma membranes (lane B), cytoplasm (lane C), endoplasmic reticulum (lane D), mitochondria (lane F), and nucleus (lane G). In lanes B and D, 25- μ g samples of protein were used, and in lanes C, F, and G, 50- μ g samples were used. Lanes A and E contain 14 C-labeled molecular weight standards and no material, respectively. (b) The following subcellular fractions from the cell-line BH were electrophoresed on a polyacrylamide gel and stained with Coomassie brilliant blue: plasma membranes (lane B), endoplasmic reticulum (lane C), mitochondria (lane D), and nucleus (lane E). Lane A contains molecular weight standards.

by immunofluorescence (see above). Cells transfected with *Bam*HI-K were shown to have stably retained and expressed the fragment by demonstrating the presence of the Epstein-Barr nuclear antigen (14, 23).

Cells. Human B cells were isolated as described previously (4, 41) and infected with EBV by incubation with undiluted supernatant from the B95-8 marmoset lymphoblastoid cell line (50% transforming dose, $>10^6$). After 2 h, an equal volume of RPMI containing 20% FCS was added. Cycloheximide (Sigma)-treated cells were preincubated for 30 min in the presence of the inhibitor before infection at a concentration of either 5 or 10 μ g/ml.

JY, ER, BH, and IARC 304 (the kind gifts of G. Lenoir) are lymphoblastoid cell lines transformed with the B95-8 isolate of EBV. RAJI and BL36 (the kind gifts of G. Lenoir) are EBV-positive Burkitt's lymphoma cell lines. RAMOS and BL2 (the kind gifts of G. Lenoir) are EBV-negative Burkitt's lymphoma cell lines. NIH 3T3 cells are mouse fibroblast cell lines (the kind gift of T. Krontiris). All lines are maintained in RPMI containing 10% FCS with penicillin and streptomycin, except JY, which is maintained on 8% FCS, and NIH 3T3, which is maintained on Dulbecco minimal essential medium containing 10% FCS. The diffuse histiocytic lymphoma cells (C6542, C6199, and C8238) and the chronic lymphocytic leukemia cells (C2146, C6248, and C2025) were the kind gifts of Lee Nadler.

RESULTS

Identification of an EBV-encoded protein in the membranes of transformed cells. We obtained three stable hybridomas, designated S6, S9, and S12, that reacted specifically with the EBV-encoded portion of the fusion protein pKH548 in an enzyme-linked immunosorbent assay. Only the S12 hybridoma produced antibody which bound protein A; therefore, this antibody has been used in all subsequent analyses. The isotype of this antibody is IgG2a. Initially, we wished to determine whether this antibody recognized a protein in EBV-transformed cells and whether it detected the native form of this protein.

Total membranes were prepared from EBV-negative and EBV-positive cells, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and analyzed by immunoblotting with the S12 monoclonal antibody. A protein with a molecular weight of approximately 63,000, hereafter referred to as p63, was recognized that was present only in the EBV-positive cells (Fig. 1). These included a Burkitt's lymphoma cell line (RAJI) and a line derived by in vitro transformation (JY). The polypeptide varied in molecular weight, being slightly smaller in RAJI (62,000) than in JY (63,000). No such polypeptides were seen with the EBV-negative Burkitt's lymphoma cell line RAMOS, and none of the cell lines reacted with the isotype-matched negative

FIG. 4. Cytofluorographic analysis of BH and RAMOS. BH (A and B) and RAMOS (C and D) cells were analyzed with the S12 antibody (thick line) and an isotype-matched negative control antibody (thin line) as live cells (B and D) or after receiving a mild permeabilizing treatment (A and C). Fluorescence increases from left to right, and cell number increases from bottom to top.

control antibody. The discrepancy in molecular weight between the polypeptides recognized in the JY and RAJI cell lines appears to be a result of variation in the number of internal repeats in the p63 gene from different isolates of EBV (22).

Immunofluorescence was used to demonstrate the reactivity of the antibodies *in situ*. Fluorescence was obtained with both the S12 and S9 antibodies by using either acetone or methanol fixation; however, stronger fluorescence was observed with the latter, which was therefore the method of choice. In Fig. 2, an EBV-positive cell line (BH) and an EBV-negative cell line (RAMOS) are shown after staining with S12. Two patterns of fluorescence were observed in BH: a bright punctate fluorescence and a diffuse cytoplasmic fluorescence. RAMOS exhibited no specific fluorescence.

Intracellular localization. Neither the immunoblotting performed upon total membranes nor the immunofluorescence on fixed cells could demonstrate the intracellular location of p63. Therefore, subcellular fractionation of the cell lines BH (EBV positive) and RAMOS (EBV negative) was performed by the procedure of Crumpton and Snary (6), and the fractions were analyzed for the presence of p63 by immunoblotting (Fig. 3a). p63 was observed in all membrane-containing fractions of the BH line: plasma membrane, endoplasmic reticular, mitochondrial, and nuclear fraction. It was not observed in the cytoplasmic fraction from BH or

in any fraction from the RAMOS cell line. The molecular weight of the protein was the same in all fractions (63,000); however, the nuclear fraction contained an additional high-molecular-weight polypeptide of approximately 120,000. The origin of the latter polypeptide is unknown; however, the molecular weight is consistent with its being a dimer. The autoradiograph was scanned with a densitometer to determine the amount of p63 in each lane. When this was normalized for the amount of protein in each sample, the relative concentrations in the plasma membrane, nuclear, mitochondrial, and endoplasmic reticular fractions were 1, 2, 1.5, and 0.5, respectively. The purity of the subcellular fractions was evaluated by staining with Coomassie brilliant blue of material electrophoresed on a polyacrylamide gel (Fig. 3b). The polypeptide patterns of the fractions were distinct, indicating that no substantial cross-contamination had occurred.

To investigate the orientation of the protein in the plasma membrane, we performed a cytofluorographic analysis on the cell lines BH and RAMOS. Intact, untreated cells labeled for analysis showed no fluorescence (Fig. 4B and D). Therefore, either the epitope was internal or it was cryptic. An extremely mild permeabilizing technique (1% Formalin, 5% sucrose) was then used as a pretreatment before antibody labeling. Positive fluorescence was seen with the EBV-positive BH cells pretreated in this way, but no positive

FIG. 5. Immunofluorescence on cells transfected with EBV genomic material. NIH 3T3 cells transfected with either the *EcoRI*-Dhet (A) or the *BamHI*-K (B) fragment of the EBV genome were analyzed with the S12 antibody by immunofluorescence.

fluorescence was seen on the EBV-negative RAMOS cells, although background fluorescence was increased after this treatment (Fig. 4A and C). This result is consistent with the carboxy half of p63 being internal but does not exclude the possibility that it is external and cryptic.

p63 is only found in cells with EBV genetic information. To demonstrate that p63 was encoded by the EBV genome, NIH 3T3 cells were transfected with plasmids containing the *EcoRI*-Dhet, *BamHI*-K, or *BamHI*-L restriction fragments along with a plasmid encoding the selectable marker SVneo (pSV2neo) or with pSV2neo alone. Positive transfectants were screened by immunofluorescence with the S12 anti-

TABLE 1. Reactivity of antibodies with various cell types

Cell type ^a	Origin	Reactivity	Method ^b
JY	EBV-LCL	+	i,f
ER	EBV-LCL	+	i,f
BH	EBV-LCL	+	i,f
IARC 304	EBV-LCL	+	i
BL 36	BL-EBV	+	i
	positive		
RAJI	BL-EBV	+	i,f
	positive		
NIH 3T3/ <i>EcoRI</i> -Dhet	Transfection	+	f
NIH 3T3/ <i>BamHI</i> -K	Transfection	—	f
NIH 3T3/ <i>BamHI</i> -L	Transfection	—	f
NIH 3T3/SVneo	Transfection	—	f
RAMOS	BL-EBV	—	i,f
	negative		
BL 2	BL-EBV	—	f
	negative		
Resting B	PBL	—	i,f
sIg ⁺ -PBL	PBL	—	f
PWM	PBL	—	f
C6542	DHL	—	i,f
C6199	DHL	—	i,f
C8238	DHL	—	i,f
C2146	B-CLL	—	i,f
C6248	B-CLL	—	i,f
C2025	B-CLL	—	i,f

^a Abbreviations: EBV-LCL, lymphoblastoid cells lines made by in vitro transformation of peripheral B cells; BL, Burkitt's lymphoma cell lines (EBV positive or negative); PBL, peripheral blood lymphocytes; sIg⁺-PBL, surface immunoglobulin-negative PBL; PWM, pokeweed mitogen-stimulated B cells; DHL, cells from diffuse histiocytic lymphomas; B-CLL, cells from B-cell-derived, surface immunoglobulin-positive chronic lymphocytic leukemias. NIH 3T3/*EcoRI*-Dhet, NIH 3T3/*BamHI*-K, NIH 3T3/*BamHI*-L and NIH 3T3/SVneo are NIH 3T3 cells transfected with *EcoRI*-Dhet, *BamHI*-K, *BamHI*-L and the plasmid containing the gene SVneo, respectively.

^b Reactivity was determined with the S12 monoclonal antibody by either immunoblotting (i) or fluorescence (f) or both methods (i,f).

body. p63 was readily detectable only in the cells transfected with the plasmid containing the *EcoRI*-Dhet fragment (Fig. 5). The pattern of fluorescence was similar to that seen in the lymphoblastoid cell lines, but the punctate speckling was particularly pronounced. We may conclude, therefore, that the *EcoRI*-Dhet fragment of the genome encodes the protein detected with the monoclonal antibodies we have developed.

To exclude the possibility that the S12 antibody also reacted with a cellular homolog of p63, we screened a variety of EBV-negative cell types by immunoblotting and immunofluorescence. These included normal activated B cells, cells from diffuse histiocytic lymphomas, a B-lymphoblastoid neoplasm, and cells from B-cell chronic lymphocytic leukemias. The latter were of interest because they express two B-cell-specific differentiation markers that are produced at high levels only on EBV-infected lymphoblastoid cell lines, Blast-1 (43) and Blast-2(EBVCS) (42). Although p63 was consistently found in EBV-positive cells, it was never detected in any EBV-negative cell types tested (Table 1). We conclude, therefore, that the S12 antibody only recognizes the EBV-encoded p63 protein.

Presence of p63 in the virion and time course of appearance in the infected cell. p63 was not observed in uninfected peripheral B cells but was always present in the EBV-transformed lymphoblastoid cell lines. We wished to identify at what time during the infection process the protein was first detected so that it could be correlated with the other events occurring early during infection (see below). Immunofluorescence was used instead of immunoblotting because with

FIG. 6. Immunofluorescence at 15 min postinfection. Cells were analyzed with the S12 antibody (A) and isotype-matched control (C). The respective bright fields (B and D) are also shown. Image enhancement techniques (see the text) were used to detect fluorescence. Uninfected B cells and infected T cells stained with the S12 antibody were uniformly as negative as panel C and are not shown.

the former, a positive reaction can be seen with a single cell, whereas immunoblotting requires 10^7 cells to detect a clear signal. Positive fluorescence was detectable at 15 min, the earliest postinfection time tested, on 15 to 20% of the cells (Fig. 6), although this may be an underestimate since the fluorescence signal was weak. It remained detectable on a

small percentage of cells until 4 h and disappeared by 15 h. The fluorescence seen at this early time was distinct from that seen in an established cell line (cf. Fig. 2 and 6). Early in infection, fluorescence was mostly restricted to the plasma membrane; however, an occasional cell showed internal staining (Fig. 6A). Although the fluorescence was

weak, it was clearly positive when compared with negative antibody controls (Fig. 6C) and to uninfected B cells (not shown). In addition, no such fluorescence was seen with infected T cells which lacked the EBV receptor (data not shown). By day 3 postinfection, 30 to 40% of the cells expressed fluorescence characteristic of that seen in established EBV-transformed cells. To rule out the possibility that the early transient expression of p63 was caused by de novo synthesis, resting B cells were incubated with the

protein synthesis inhibitor cycloheximide before infection with EBV. The treated cells expressed the same level of fluorescence that was seen in untreated cells at the same early time point (data not shown). These results indicate that the protein was present in the virion. This was confirmed when we analyzed highly purified virions by immunoblotting with the anti-p63 monoclonal antibody (Fig. 7a). p63 was readily detected in virion preparations, and the signal was of similar intensity to that seen when the same amount of

FIG. 7. Immunoblot on purified virion. (a) Purified EBV (lanes A and C) and a plasma membrane preparation of IARC304 (EBV positive) (lanes B and D) were analyzed with both the S12 monoclonal antibody (lanes A and B) and an isotype-matched negative control antibody (lanes C and D). (b) [^{35}S]methionine-labeled virions and total membrane preparations of B95-8 cells: total membrane preparation B95-8 (lane A), detergent-soluble material from B95-8 (lane B), whole virions (lane C), and detergent-soluble material from whole virions (lane D).

protein from purified plasma membranes was used. Reactivity with a 45,000-dalton polypeptide was also seen in this experiment. Although this is a specific reaction (it was not seen with the control antibody [Fig. 7a, lanes C and D]), it did not occur reproducibly, and its origin is unclear. The virion preparation is highly enriched for the specific virus proteins and does not contain significant levels of contamination by proteins from cellular membranes (Fig. 7b). These results are consistent with the theory that p63 is carried in with the virion, is inserted into the plasma membrane concomitant with infection, disappears, and is synthesized de novo upon transformation.

DISCUSSION

This report contains the first demonstration of an EBV-encoded protein found in the plasma membrane of EBV-transformed cells. The 63,000-molecular-weight protein, p63, is encoded by the *EcoRI*-Dhet fragment of the genome, and the mRNA from this region is the most abundant viral RNA found in EBV-transformed cells. It is of interest that p63 must also be synthesized in the productively infected cell since it is a virion component. The transient appearance of p63 immediately after infection (a direct result of the presence of the protein in the virion) suggests that it may

play an active role in the initial events of transformation by EBV.

Lymphocyte-determined membrane antigen, the unidentified target antigen of cytotoxic T cells directed against EBV-infected cells, must be located on the plasma membrane since cytotoxic T cells recognize and lyse live untreated cells. Therefore, p63 may be the lymphocyte-determined membrane antigen. The observation that the monoclonal antibodies described in this report do not react with the surface of untreated cells is not inconsistent with this possibility since the fusion protein pKH548 used to generate the antibodies contained only the carboxy half of p63, and the cytotoxic T cells could recognize a structure elsewhere on the polypeptide.

To detect the 63,000-molecular-weight protein, some form of permeabilization of the cells was necessary. This is consistent with the carboxy terminus being internal; however, we cannot eliminate the possibility that the epitopes recognized by the monoclonal antibodies are on the cell surface but are cryptic and are exposed by the fixation procedure.

We have previously proposed that EBV-mediated transformation is a two-step process (43). First, the resting B cell is infected and driven to differentiate to the lymphoblastoid stage. Second, the cell is fixed at this stage and remains a

continuously proliferating lymphoblast. Within the context of this model, the presence of p63 immediately after infection suggests that it could provide part of an early activation signal for the B cell. It is worth noting that the predicted secondary structure of p63 is similar to that of known ion channels (13) and that calcium alone is sufficient to activate B cells (for a review, see reference 47). In addition, it has recently been shown that there is an early calcium-dependent event during EBV transformation (31). Therefore, p63 could provide part of an early activation signal by acting as an ion channel. p63 alone is not sufficient for B-cell activation, since irradiated virions will not activate B cells, implying a requirement for an intact EBV genome (18).

Newly synthesized p63 was not detectable until day 3 postinfection, the time when the cells undergo blast transformation. This is distinct from the other well-characterized EBV transformation antigen Epstein-Barr nuclear antigen (12, 36, 39), which is synthesized within a few hours after infection. EBV-infected cells are known to have a decreased serum requirement for calcium (32) after the transformation event; it is conceivable that p63 could also act as an ion channel to help maintain the transformed state.

It has recently been shown that at least two oncogenes are required for transformation of primary cells (28). The two oncogenes must come from complementary groups, and for the most part, members of one group are associated with nuclear structures, and members of the complementary group are associated with the plasma membrane (for a review, see reference 28). The protein p63 could have a role analogous to that of proteins of the latter group which includes among others ras, src, abl, erb-B, and polyoma middle T, perhaps cooperating with Epstein-Barr nuclear antigen 1 or 2 or both to achieve transformation.

Although p63 was found in highly purified plasma membranes, it was also found in all other membrane fractions. This presence is probably not a result of cross-contamination between fractions since a distinct pattern of polypeptides is seen in each fraction when stained with Coomassie brilliant blue. Similarly, the [³⁵S]methionine-labeled virion preparation indicates considerable enhancement of virion components and little detectable contamination from cellular membranes. In addition, since viral envelopes do not include a random assortment of proteins, it is also unlikely that the presence of p63 in the virion is a result of nonspecific acquisition during budding.

While this manuscript was in preparation, Hennessy et al. (22) described a rabbit serum against the pKH548 fusion protein and showed that it reacted with a 55,000- to 60,000-molecular-weight protein present in unfractionated cellular membrane preparations, but absent from the cytoplasm. Our results agree with those of Hennessy et al. and extend these observations to show that the protein is present in all membrane fractions but, most importantly, is in highly purified plasma membranes and purified virion preparations.

Hennessy et al. further proposed that the discrepancy between the predicted molecular weight of 42,000 and the observed weight of 63,000 is due to anomalous migration in sodium dodecyl sulfate-polyacrylamide gel electrophoresis and that the slight variability in the molecular weight of the protein between different cell lines is a result of different numbers of direct repeats present in the DNA sequence (22).

In addition to p63, a smaller protein was recognized in the virion preparation and in some membrane preparations. This 45,000-molecular-weight protein may be the product of a second smaller RNA from this region which is transcribed during productive infections (24).

The availability of monoclonal antibodies specific for p63 will allow investigations of the role of this protein in EBV infection and specifically will help to elucidate whether p63 is involved in the transformation process or T-cell-mediated immunity or both.

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