Orientation and Patching of the Latent Infection Membrane Protein Encoded by Epstein-Barr Virus

DAVID LIEBOWITZ, DAVID WANG, AND ELLIOTT KIEFF*

Kovler Viral Oncology Laboratories, The University of Chicago, Chicago, Illinois 60637

Received 1 August 1985/Accepted 23 December 1985

Epstein-Barr virus is known to encode three nuclear proteins and one membrane protein (LMP) in latently infected growth-transformed cells. Studies of the plasma membrane localization and orientation of LMP by protease digestion of live cells and by immunofluorescence indicated the following. (i) At least 30% of LMP is in the plasma membrane, as opposed to other cytoplasmic membranes. (ii) A small LMP domain which corresponds to a previously proposed outer reverse turn between the first two transmembrane domains is exposed on the outer cell surface (and two other proposed outer-reverse-turn domains may be exposed), whereas all or almost all of the rest of the protein is not exposed on the outer cell surface. (iii) LMP is present in patches in the cell plasma membrane.

Epstein-Barr virus (EBV) is a likely etiologic agent of African Burkitt's lymphoma and of nasopharyngeal carcinoma (for a review, see reference 3). EBV infection is, however, prevalent in all human populations. The virus infects human B lymphocytes and can be persistently found in these cells in a latent state (8, 16, 17). As a consequence of latent virus infection, the cells proliferate indefinitely in cultures and can also proliferate in immunodeficient humans (for a review, see reference 18). The virus encodes three nuclear proteins and one membrane protein (LMP) in latently infected cells (4, 9-12; for a review, see reference 13 and T. Dambaugh, K. Hennessy, S. Fennwald, and E. Kieff, in M. A. Epstein and B. G. Achong, ed., *The Epstein-Barr Virus: Recent Advances*, in press). LMP may play a role in viral stimulation of cell proliferation (30) and may also be the target antigen (LYDMA) recognized by those immune T lymphocytes which specifically kill latently infected B lymphocytes (15, 25).

The existence of an EBV-encoded membrane protein was first postulated from an analysis of the predicted amino acid sequence of an open reading frame of an mRNA from latently infected cells (4; Fig. 1). The relevant characteristics of the predicted primary amino acid sequence include the following: (i) a hydrophilic amino terminus not characteristic of signal peptides (29), (ii) six markedly hydrophobic 20-amino-acid segments separated from each other by five less hydrophobic 8-amino-acid segments, an arrangement characteristic of contiguous membrane insertion sequences (24), (iii) a 210-amino-acid hydrophilic and acidic carboxy-terminal domain, and (iv) the absence of potential N-linked glycosylation sites. On the basis of these characteristics, a model for the possible orientation of the protein in the plasma membrane was proposed (4; Fig. 1). The hydrophilic carboxy half of the protein was subsequently synthesized as a stable beta-galactosidase fusion protein in *Escherichia coli* (9; Fig. 1). Antibody to the purified fusion protein demonstrated that an EBV protein of the expected size was associated with the cytoplasmic membranes of latently infected cells (9). Further, in immunofluorescence microscopy the antisera detected a rim of antigen at the margin of the cytoplasm of fixed and permeabilized, but not live, lymphocytes, indicating that the carboxy part of the protein was in

the inner aspect of the plasma membrane (9). Subsequently, a monoclonal antibody, S12, against the EBV LMP-beta-galactosidase fusion protein (14) was derived. The monoclonal antibody reacted with plasma membrane, endoplasmic reticulum, and nuclear membrane cell fractions partially purified by differential centrifugation (14). However, in these studies, the extent of purity of the various cell membrane fractions was not directly assessed.

The location and orientation of the protein in the plasma membrane are of considerable significance in the role of the protein in T-cell recognition and in cell transformation (4, 9, 30). We therefore undertook a series of experiments to validate the proposed model for the orientation of the protein in the plasma membrane.

In the first experiments, the extent of exposure of LMP on the outer cell surface was evaluated by determining the sensitivity of the protein to proteases which should cleave the protein at multiple specific sites (Fig. 1) if those sites were exposed on the outer cell surface. Intact live cells were treated with trypsin or chymotrypsin and the size of the residual LMP was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and immunoblotting with monoclonal antibody S12, which reacts with an epitope in the carboxy-terminal half of the protein. The results (Fig. 2A) indicated that chymotrypsin reduces the size of about 30% of LMP from 60 to 55 kilodaltons (kDa), indicating that there is an exposed chymotrypsin site approximately 50 amino acids from one end of the molecule. There is one such site at the first outer reverse turn 47 amino acids from the amino terminus (Fig. 1). Cleavage at this site would reduce the size of LMP by 6.3 kDa. Other potential chymotrypsin sites between the amino terminus and the first outer reverse turn would, if cleaved, result in a longer residual LMP than was observed. These sites are also in a very hydrophobic domain, highly unlikely to be exposed on the outer cell surface (Fig. 1). There is no chymotrypsin site within the carboxy terminus which could reduce the protein to a comparable size. The cleavage of LMP by chymotrypsin was not an artifact of intracellular penetration of chymotrypsin, since actin was not cleaved (Fig. 2B). The proportion of cleaved LMP did not increase with increasing chymotrypsin concentration or time of digestion (Fig. 2A). Increased chymotrypsin digestion times, but not increased chymotrypsin concentrations, resulted in minor LMP digestion prod-

* Corresponding author.

FIG. 1. Proposed model for the insertion of EBV LMP into the cell plasma membrane. The cytoplasmic surface of the cell is shown below the phospholipid bilayer. Potential trypsin and chymotrypsin cleavage sites are indicated by open and filled arrows, respectively. The boxed area indicates the part of the protein which was fused to beta-galactosidase and expressed in *E. coli*. The fusion protein was used to immunize mice for the generation of monoclonal antibody S12.

ucts smaller than 55 kDa (Fig. 2A, lanes CT 2 and CT 4). These minor fragments could be due to chymotrypsin cleavage at sites in the second and third outer-reverse-turn domains, since the sizes of the minor fragments were 52, 44, and 42 kDa, whereas the expected sizes of the cleavage products were 49, 42, and 41 kDa, respectively.

Although there are multiple trypsin cleavage sites in the amino- and carboxy-terminal domains (Fig. 1), trypsin treatment of intact live cells had no effect on LMP (Fig. 3A). Even a brief trypsin treatment resulted in extensive degradation of HLA class 1, a protein known to be exposed on the outer cell surface (Fig. 3B). As expected from the lack of digestion of LMP by trypsin, there was also usually no actin degradation (data not shown). To demonstrate that LMP is sensitive to trypsin and chymotrypsin digestion, we exposed LMP purified by immunoaffinity chromatography to 0.01% of the protease concentrations used in these experiments. Even at the lowest protease concentrations, LMP was degraded beyond the level of detection in the immunoblot analysis used in Fig. 2 and 3. Since the amino-terminal, carboxy-terminal, and proposed inner-reverse-turn domains of LMP have trypsin cleavage sites and are not degraded by trypsin treatment of live cells, these domains are not likely to be exposed on the outer cell surface.

These experiments indicated that (i) at least 30% of the cytoplasmic LMP is in the plasma membrane, as opposed to other membrane compartments of the cell, (ii) the amino-

terminal, carboxy-terminal, and proposed inner-reverse-turn domains are not on the outside of the plasma membrane, and (iii) the first outer reverse turn is sufficiently exposed on the cell surface to be susceptible to protease cleavage. Thus, these results strongly support the proposed model for the orientation of LMP in the cell membrane. Although significant chymotrypsin digestion of the proposed second and third outer-reverse-turn domains was not observed despite potential chymotrypsin cleavage sites, it should be noted that these sites are within the hydrophobic domain at the junction with hydrophilic amino acids and are therefore less likely to protrude sufficiently out of the membrane to be susceptible to chymotrypsin treatment of live cells. In contrast, the chymotrypsin site which was cleaved in the first outer reverse turn is directly preceded by an aspartic acid and followed by a threonine. By the same rationale, the trypsin site in the first inner reverse turn and the chymotrypsin site in the second inner reverse turn are also likely to protrude out of the membrane. Thus, their insusceptibility to these proteases is more significant than that of the second and third outer-reverse-turn domains. An important implication of the demonstration that a part of LMP is exposed on the outer cell surface is that LMP could be all or part of LYDMA. If so, the outer-reverse-turn domains of LMP are likely candidates for T-cell recognition.

Monospecific rabbit anti-LMP sera and monoclonal antibody S12 are directed against the hydrophilic carboxy half of

FIG. 2. Cell surface chymotrypsin proteolysis of lymphocytes latently infected with EBV. Jijoye cells (2×10^7) grown at >95% viability were washed once in phosphate-buffered saline (PBS) and incubated in PBS with no protease for 30 min at 37°C (CON), incubated in PBS with 200 U of chymotrypsin for 30 min at 37°C (CT 1), incubated in PBS with 200 U of chymotrypsin for 60 min at 37°C (CT 2), incubated in PBS with 400 U of chymotrypsin for 30 min at 37°C (CT 3), or incubated in PBS with 400 U of chymotrypsin for 60 min at 37°C (CT 4). Samples were treated as described in the legend to Fig. 3A unless otherwise specified. Equal amounts of protein were then electrophoresed on an 8% sodium dodecyl sulfate-polyacrylamide gel and transferred to nitrocellulose. In Panel A, the nitrocellulose was immunostained with monoclonal antibody S12. In panel B, CON cell lysate and CT 1 cell lysate were immunostained with an anti-actin monoclonal antibody (Amersham Corp.). Similar results were obtained with IB4 cells. Numbers at right indicate sizes in kilodaltons.

LMP and did not react with live cells (9, 14; data not shown), confirming the intracytoplasmic orientation of this LMP domain. To investigate the distribution of LMP in cells, we fixed latently infected cells with methanol and stained them with monoclonal antibody S12. Several types of fluorescence (Fig. 4A to D) were seen in latently infected IB4 cells or in Jijoye cells which were predominantly latently infected. (About 2% of the Jijoye cells showed evidence of early or late viral replicative antigens.) Most IB4 or Jijoye cells had a discontinuous rim of punctate fluorescence which surrounded most or all of the outer cell margin (Fig. 4A and D), lighter and more diffuse fluorescence over the cell cytoplasm (Fig. 4A), and one or more patches of intense fluorescence at the cell margin (Fig. 4A to C). The rim of punctate fluorescence was difficult to photograph, since the entire rim could not be brought into simultaneous sharp focus. The patches of discrete intense rim fluorescence did not bear any consistent relationship to the diffuse cytoplasmic fluorescence. A rim of nuclear membrane fluorescence could not be distinguished. These results indicated that LMP is present in patches in the plasma membrane of latently infected cells. The patching is not an artifact of fixation, since a monoclonal antibody to

FIG. 3. Cell surface trypsin proteolysis of lymphocytes latently infected with EBV. (A) Jijoye cells (2×10^7) grown at >95% viability were washed once in phosphate-buffered saline (PBS) and split into two equal samples. One sample (TRY) was treated with 200 U of tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Worthington Diagnostics) for 30 min at 37°C, and the other sample (CON) was incubated in PBS with no protease for 30 min at 37°C. Both samples were then washed two times in PBS with 1 mM phenylmethylsulfonyl chloride (PMSF) and 1 mM TPCK. The cells were then solubilized in SDS sample buffer, and equal amounts of protein were run on a 7% SDS-polyacrylamide gel and transferred to nitrocellulose for immunostaining with monoclonal antibody S12, rabbit anti-mouse immunoglobulin G (Cooper Biomedical, Inc.), and ^{125} I-protein A (Amersham). (B) Jijoye cells (10^7) were surface labeled with 0.5 mCi of Na^{125}I (Amersham) for 15 min by using 10 μg of Iodogen (Pierce Chemical Co.). The cells were washed twice in PBS and split into two equal samples. One sample (TRY) was treated with 200 U of TPCK-treated trypsin for 1 min at 25°C, and the other sample (CON) was not treated with protease. The cells were lysed in 50 mM Tris hydrochloride (pH 7.0)–120 mM NaCl–0.5% (vol/vol) Nonidet P-40–1 mM PMSF–1 mM TPCK for 30 min on ice and clarified by centrifugation in an Eppendorf microcentrifuge for 1 min at 4°C. The extracts were immunoprecipitated with W6/32 (a monoclonal antibody which recognizes HLA class 1 antigen [1]) for 2 h on ice and collected by adsorption to Formalin-fixed *Staphylococcus aureus* Cowan 1 (Calbiochem-Behring). After three washes, the immunoprecipitates were eluted in SDS sample buffer, and equal amounts of protein were analyzed on an 8% SDS-polyacrylamide gel. Numbers at the right indicate sizes in kilodaltons.

HLA class 1 protein demonstrated its diffuse distribution on the surface of methanol-fixed cells (Fig. 4E). Furthermore, fixation of cells with acetone, glutaraldehyde, formaldehyde, periodate-lysine-paraformaldehyde, or ethanol and reaction with monoclonal antibody S12 yielded results similar to

FIG. 4. Fluorescent microscopy of IB4 and Jijoye cells. Concentrated Jijoye (A, B, C, and E) or IB4 (D) cells were dried on a glass slide for 30 min at 37°C, fixed with methanol at -20°C for 6 min, washed two times in phosphate-buffered saline with 10% goat serum, incubated with monoclonal antibody S12 (A to D) or with anti-HLA monoclonal antibody W6/32 (E) for 1.5 h at 25°C, washed, and incubated with biotin-conjugated goat anti-mouse immunoglobulin G (1:100 dilutions) for 1 h at 25°C and with fluorescein isothiocyanate-conjugated streptavidin for 30 min at 25°C. The cells were photographed at a magnification of $\times 408$ (A) or $\times 816$ (B, C, D, and E).

those obtained with cells fixed with methanol (data not shown).

Taken together, these data provide strong evidence that a substantial fraction of EBV LMP is in the cell plasma membrane, as was proposed previously (4, 9, 14), and that LMP aggregates in punctate and large patches in the membrane, as is characteristic of receptor molecules (19–21, 31). Since it is likely that only six or seven amino acids of the outer reverse turns are exposed on the cell surface membrane (Fig. 1), it is unlikely that LMP is itself a receptor. LMP could, however, play an accessory role to a cell growth factor receptor in promoting the growth of latently infected cells. EBV-infected cells have been shown to secrete and respond to B-cell growth factors (2, 5–7). The patching of

LMP may occur because of an association with a cell growth factor receptor. The expression of LMP in rodent cells results in altered cell morphology and resistance to the growth-inhibiting effects of media containing low serum. LMP also induces a loss of contact inhibition and anchorage independence in Rat-1 cells and causes Rat-1 cells to be tumorigenic in nude mice (30). LMP resembles rhodopsin in that both molecules have multiple membrane insertion domains but differs in that both the amino and carboxy termini of LMP are cytoplasmic, whereas only the carboxy terminus of rhodopsin is cytoplasmic, the amino terminus being located on the opposite side of the membrane. Although rhodopsin diffuses freely in the membrane and does not form patches, recent data suggest that rhodopsin may transduce through G-protein activation (for a review, see reference 22). GTP-binding proteins such as the *ras* family of oncogenes are clearly important in the regulation of cell growth (23, 26–28).

This work was facilitated by the development of monoclonal antibody S12 by K. Mann and D. Thorley-Lawson, by the gift of anti-HLA monoclonal antibody W6/32 from J. Weis and D. Fearon, and by the advice of F. Kezdy and J. Tanner.

The research was supported by Public Health Service grants CA 17281 and CA 19264 from the National Cancer Institute. David Liebowitz and David Wang are predoctoral trainees supported by Public Health Service grant GM 07281 from the National Institutes of Health and grant IIE 15822181 from the Institute of International Education, respectively.

LITERATURE CITED

1. Barnstable, C. J., W. F. Bodmer, G. Brown, G. Galfre, C. Milstein, A. E. Williams, and A. Ziegler. 1978. Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens—new tools for genetic analysis. *Cell* 14:9–20.
2. Blazar, B. A., L. M. Sutton, and M. Strome. 1983. Self-stimulating growth factor production by B-cell lines derived from Burkitt's lymphomas and other lines transformed in vitro by Epstein-Barr virus. *Cancer Res.* 43:4562–4568.
3. de The, G. 1982. Epidemiology of Epstein-Barr virus and associated diseases in man, p. 25–103. In B. Roizman (ed.), *The herpesviruses*. Plenum Publishing Corp., New York.
4. Fennewald, S., V. van Santen, and E. Kieff. 1984. Nucleotide sequence of an mRNA transcribed in latent growth-transforming virus infection indicates that it may encode a membrane protein. *J. Virol.* 51:411–419.
5. Gordon, J., P. Aman, A. Rosen, I. Ernberg, B. Ehlin-Henriksson, and G. Klein. 1985. Capacity of B-lymphocyte lines of diverse tumor origin to produce and respond to B-cell growth factors: a progression model for B-cell lymphomagenesis. *Int. J. Cancer* 35:251–256.
6. Gordon, J., S. C. Ley, M. D. Melamed, P. Aman, and N. C. Hughes-Jones. 1984. Soluble factor requirements for the autostimulatory growth of B lymphoblasts immortalized by Epstein-Barr virus. *J. Exp. Med.* 159:1554–1559.
7. Gordon, J., S. C. Ley, M. D. Melamed, L. S. English, and N. C. Hughes-Jones. 1984. Immortalized B lymphocytes produce B-cell growth factor. *Nature (London)* 310:145–147.
8. Henle, W., B. Diehl, G. Kohn, H. zur Hausen, and G. Henle. 1967. Herpes-type virus and chromosome marker in normal leukocytes after growth with irradiated Burkitt cells. *Science* 157:1065–1075.
9. 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.
10. Hennessy, K., S. Fennewald, and E. Kieff. 1985. A third nuclear protein in lymphoblasts immortalized by Epstein-Barr virus. *Proc. Natl. Acad. Sci. USA* 82:5944–5948.

11. Hennessy, K., and E. Kieff. 1983. One of two Epstein-Barr virus nuclear antigens contains a glycine-alanine copolymer domain. *Proc. Natl. Acad. Sci. USA* **80**:5665-5669.
12. Hennessy, K., and E. Kieff. 1985. A second nuclear protein is encoded by Epstein-Barr virus in latent infection. *Science* **227**:1238-1240.
13. Kieff, E., K. Hennessy, S. Fennewald, T. Matsuo, T. Dambaugh, M. Heller, and M. Hummel. 1985. Biochemistry of latent Epstein-Barr virus infection and associated cell growth transformation, p. 323-339. *In* G. Lenoir, G. O'Connor, and C. Olweny (ed.), *Proceedings of Burkitt's lymphoma: a human cancer model*. International Agency for Research on Cancer, Lyon, France.
14. Mann, K. P., D. Staunton, and D. A. Thorley-Lawson. 1985. An Epstein-Barr virus-encoded protein found in plasma membranes of transformed cells. *J. Virol.* **55**:710-720.
15. Moss, D., L. Wallace, A. Rickinson, and M. Epstein. 1981. Cytotoxic T-cell recognition of Epstein-Barr virus infected B-cells. I. Specificity and HLA restriction of effector cells reactivated in vitro. *Eur. J. Immunol.* **11**:686-693.
16. Nilsson, K. 1971. High frequency establishment of human immunoglobulin producing lymphoblastoid lines from normal and malignant lymphoid tissue and peripheral blood. *Int. J. Cancer* **8**:432-442.
17. Pope, J. H., M. K. Horne, and W. Scott. 1968. Transformation of fetal human leucocytes in vitro by filtrates of a human leukemic cell line containing herpes-like virus. *Int. J. Cancer* **3**:857-866.
18. Purtilo, D., G. Manolov, E. Tatsumi, Y. Manolova, S. Harada, H. Lipscomb, and G. Krueger. 1985. Epstein-Barr virus and the pathogenesis of lymphoproliferative and aroliferative diseases in immune deficient patients. *Int. Rev. Exp. Pathol.* **27**:113-183.
19. Schechter, Y., L. Hernaez, J. Schlessinger, and P. Cuatrecasas. 1979. Local aggregation of hormone-receptor complexes is required for activation by epidermal growth factor. *Nature (London)* **278**:835-839.
20. Schlessinger, J., Y. Schechter, M. C. Willingham, and I. Pastan. 1978. Direct visualization of binding, aggregation, and internalization of insulin and epidermal growth factor on living fibroblastic cells. *Proc. Natl. Acad. Sci. USA* **75**:2659-2663.
21. Schreiber, A. B., T. A. Libermann, I. Lax, Y. Yarden, and J. Schlessinger. 1983. Biological role of epidermal growth factor-receptor clustering. Investigation with monoclonal anti-receptor antibodies. *J. Biol. Chem.* **258**:846-853.
22. Schwartz, E. 1985. Phototransduction in vertebrate rots. *Annu. Rev. Neurosci.* **8**:339-367.
23. Shih, T., A. Papageorge, P. Stokes, M. Weeks, and E. Scolnick. 1980. Guanine nucleotide binding and autophosphorylating activities associated with the p21 src protein of Harvey murine sarcoma virus. *Nature (London)* **287**:686-691.
24. Silhavy, T. J., S. A. Benson, and S. D. Emr. 1983. Mechanisms of protein localization. *Microbiol. Rev.* **47**:313-344.
25. Svedmyr, E., and M. Jondal. 1975. Cytotoxic effector cells specific for B cell lines transformed by Epstein-Barr virus are present in patients with infectious mononucleosis. *Proc. Natl. Acad. Sci. USA* **72**:1622-1666.
26. Tabin, C., S. Bradley, C. Bargmann, R. Weinberg, A. Papageorge, E. Scolnick, R. Dhar, D. Lowy, and E. Chang. 1982. Mechanism of activation of a human oncogene. *Nature (London)* **300**:143-148.
27. Tatchell, K., D. Chaleff, D. Defeo-Jones, and E. Scolnick. 1984. Requirement of either of a pair of ras-related genes of *Saccharomyces cerevisiae* for spore viability. *Nature (London)* **309**:523-527.
28. Toda, T., I. Uno, T. Ishikawa, S. Powers, T. Kataoka, D. Broek, S. Cameron, J. Broach, K. Matsumoto, and M. Wigler. 1985. In yeast, Ras proteins are controlling elements of adenylate cyclase. *Cell* **40**:27-36.
29. Van Heijne, G. 1983. Patterns of amino acids near signal sequence cleavage sites. *Eur. J. Biochem.* **133**:17-21.
30. Wang, D., D. Liebowitz, and E. Kieff. 1985. An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells. *Cell* **43**:831-840.
31. Zidovetzki, R., Y. Yarden, J. Schlessinger, and T. M. Jovin. 1981. Rotational diffusion of epidermal growth factor complexed to cell surface receptors reflects rapid microaggregation and endocytosis of occupied receptors. *Proc. Natl. Acad. Sci. USA* **78**:6981-6985.