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The Epstein-Barr Virus Latent Membrane Protein 1 Induces Expression of the Epidermal Growth Factor Receptor

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The Epstein-Barr virus (EBV)-encoded LMP1 protein is an important component of the process of transformation by EBV. LMP1 is essential for transformation of B lymphocytes, most likely because of its profound effects on cellular gene expression. Although LMP1 is expressed in the majority of nasopharyngeal carcinoma (NPC) tumors, the effect of LMP1 on cellular gene expression and its contribution to the development of malignancy in epithelial cells is largely unknown. In this study the effects of LMP1 on the expression and tyrosine kinase activity of the epidermal growth factor receptor (EGFR) were investigated in C33A human epithelial cells. Stable or transient expression of LMP1 in C33A cells increased expression of the EGFR at both the protein and mRNA levels. In contrast, expression of the EGFR was not induced by LMP1 in EBV-infected B lymphocytes. Stimulation of LMP1-expressing C33A cells with epidermal growth factor (EGF) caused rapid tyrosine phosphorylation of the EGFR (pp170) as well as several other proteins, including pp120, pp85, pp75, and pp55, indicating that the EGFR induced by LMP1 is functional. LMP1 also induced expression of the A20 gene in C33A epithelial cells. In C33A cells, LMP1 expression increased the proliferative response to EGF, as LMP1-expressing C33A cells continued to increase in number when plated in serum-free media supplemented with EGF, while the neo control cells exhibited very low levels of viability and did not proliferate. Immunoblot analysis of protein extracts from nude mouse-passaged NPC tumors also demonstrated that the EGFR is overexpressed in primary NPC tumors as well as those passaged in nude mice. This study suggests that the alteration in the growth patterns of C33A cells expressing LMP1 is a result of increased proliferative signals due to enhanced EGFR expression, as well as protection from cell death due to LMP1-induced A20 expression. The induction of EGFR and A20 by LMP1 may be an important component of EBV infection in epithelial cells and could contribute to the development of epithelial malignancies such as NPC.

Epstein-Barr virus (EBV), a ubiquitious human herpesvirus, is consistently detected in several human malignancies, including endemic Burkitt's lymphoma (BL), nasopharyngeal carcinoma (NPC), and posttransplant lymphoma (32, 38, 46, 63). EBV readily infects and transforms human B lymphocytes in culture; however, the lack of a suitable epithelial cell infection system has hindered studies of the mechanisms leading to transformation of this cell type by EBV. Transformation of B lymphocytes in vitro requires expression of four nuclear antigens (EBNA1, -2, -3A, and -3C) as well as the latent membrane protein 1 (LMP1) (8, 30, 56).

Analysis of EBV gene expression in biopsies of the human epithelial malignancy NPC has revealed that viral gene expression is limited to EBNA1, LMP1, LMP2, and a family of rightward transcripts from the *Bam*HI A region of the EBV genome (4, 17, 18, 25). The latent origin binding protein EBNA1 has been detected in 100% of tumor biopsies, while the LMP1 protein has been detected in approximately 60% of tumor biopsies (14, 64). Analysis of carcinoma in situ or preinvasive lesions of the nasopharynx has revealed expression of LMP1 protein in 100% of the cases, suggesting an important role for LMP1 in the development of NPC (47).

Biochemical studies of the effect of LMP1 expression in B lymphocytes have shown that LMP1 induces the expression of the Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase-Gr), the B-cell activation antigen CD23, and adhesion molecules ICAM-1, LFA-1, and LFA-3 as well as several molecules with antiapoptotic properties, including A20 and bcl-2 (24, 34, 41, 61). The requirement for LMP1 expression in the transformation of B lymphocytes likely involves an effect on these molecules. LMP1 also has transforming properties in rodent fibroblasts, indicating that it can induce cellular changes in cell types other than lymphocytes (1, 39, 60). Several studies have suggested that LMP1 can also affect growth and differentiation of immortalized, nontumorigenic epithelial cells in vitro, but they did not reveal how these changes in growth control may be mediated (9, 15).

The epidermal growth factor receptor (EGFR) is a membrane-bound glycoprotein with an extracellular ligand binding domain and an intracellular cytoplasmic domain which upon binding of epidermal growth factor (EGF) possesses an activated intrinsic tyrosine kinase domain (6, 12, 57). Stimulation of EGFR tyrosine kinase activity produces receptor autophosphorylation, which mediates interaction with SH2 and SH3 binding proteins. This in turn results in transcription factor activation and DNA replication (44). An important pathway activated by the EGFR tyrosine kinase is the Ras pathway, mediated by the SH2-containing Shc and Grb2 proteins, which through interaction with guanine nucleotide exchange factors results in activation of Ras by increasing the level of bound GTP (2, 3).

Increased expression of the EGFR gene is often associated with transformation of cells of epithelial origin, and a myriad of human epithelial tumors, including NPC, overexpress the EGFR (52, 65). Immunohistochemical analysis of tissue from these tumors in comparison to surrounding normal tissue dem-

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onstrated an elevated level of the EGFR protein in the tumor tissue. Since EBV is an important factor in the development of NPC, it was important to determine whether a viral gene product induced expression of the EGFR, leading to a deregulation of cellular growth control. The effect of LMP1 expression in rodent fibroblasts or human squamous carcinoma cells resembles that of activated Ras molecules, suggesting a common biochemical pathway for these oncoproteins (9, 60). Since overexpression or activation of the EGFR by LMP1 could mimic an activated Ras pathway, LMP1 may exert its transforming effects in epithelial cells through deregulation of the EGFR.

In this study, the EBV-encoded LMP1 gene was expressed in several established epithelial cell lines. The data indicate that LMP1 induces expression of the EGFR at both the protein and mRNA levels in the cervical carcinoma cell line C33A. Expression of LMP1 in C33A epithelial cells also resulted in increased expression of the A20 gene, which has been shown to block apoptosis in experimental systems (42). When the LMP1-transfected cells were grown in serum-free media supplemented with EGF, the cells exhibited increased viability, suggesting that the LMP1 derivatives had a growth advantage over the control cells. These results suggest that LMP1 may exert its transforming properties in epithelial cells in part through the increased expression of the cellular EGFR and A20 oncogenes.

MATERIALS AND METHODS

Cell lines and NPC tumors. C33A and SiHa epithelial cells derived from human cervical carcinomas were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM-H) supplemented with 10% fetal calf serum (Irvine Scientific) and antibiotics. HT29 cells derived from a colonic carcinoma and the cervical carcinoma-derived ME-180 cell line were grown as described above except with McCoys 5A medium. Cells were routinely grown in 100-mm-diameter cell culture dishes and split 1:5 three times weekly. Lymphoid cell lines were maintained in suspension at between 2×10^5 and 1×10^6 cells per ml in RPMI 1640 supplemented with 10% fetal calf serum and antibiotics. C33A cells expressing LMP1 were obtained by selection in 600 μg of G418 (Gibco) per ml following electroporation of an expression construct containing the LMP1 gene (B95-8 origin) driven by a human K14 promoter (LMP1 clones 1C1, 2C, and 1B4 and pools P4, P5, and P6) (27). The pGEM-based plasmid also contains the Neo^r cassette from pMAMneo (Clontech). neo control cell lines were derived either with the pMAMneo plasmid (neo pools P1, P2, and P3) or with the same construct from which the promoter and LMP1 sequences were removed by restriction digestion (neo clones 1C, 1B, and 1A2). ME-180 and HT29 cells expressing the LMP1 gene and relevant controls were constructed as described above. C15 and C17 are EBV-positive NPC tumors which have been passaged serially in nude mice.

Immunoblot analysis of protein levels. Protein extracts were made by directly scraping cells from tissue culture dishes at ~80% confluence into radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 1% deoxycholate with protease inhibitors) with SDS sample buffer (C33A cells) or by resuspending 10^6 cells in phosphate-buffered saline (PBS) before adding equal volumes of 2× SDS sample buffer (lymphoid cells). EGFR expression in NPC tumors was analyzed by using crude membrane preparations. Tumors were harvested from nude mice, disrupted in a microdismembrator, and subjected to Dounce homogenization. Unlysed cells and nuclei were removed by low-speed centrifugation, and crude membranes were pelleted at high speed. Protein (100 μg) from crude membranes was suspended in PBS and SDS sample buffer. Extracts were boiled for 10 min, separated on SDS–7% polyacrylamide gels, and transferred to supported nitrocellulose filters (Schleicher and Schuell) by using a Hoefer Semi-Dry transfer apparatus. Filters were stained with Ponceau S stain to ensure equal loading of protein in all lanes. Nonspecific reactivity was blocked by incubation overnight in Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat dried milk (BLOTTO). Supernatant from the S12 monoclonal antibody (a generous gift of David A. Thorley-Lawson) was used at a 1:10 dilution in BLOTTO for the detection of LMP1. A rabbit antiserum raised against the carboxy-terminal 100 amino acids of the EGFR fused to glutathione S-transferase (ERCT) was used at a 1:1,500 dilution in BLOTTO for the detection of the EGFR. The anti-phosphotyrosine PT-66 antibody (Sigma) was used at a 1:2,000 dilution in BLOTTO for the detection of phosphotyrosine-containing proteins. Appropriate secondary anti-mouse or anti-rabbit antibodies (Amersham) were used at a dilution of 1:2,000 in BLOTTO to detect bound primary antibody. Reactive proteins were detected by incubation of washed filters in the enhanced chemiluminescence system (ECL; Amersham) followed by exposure to autoradiographic film.

Analysis of EGFR upregulation in a transient assay. C33A cells were trypsinized at subconfluence, counted, and resuspended in DMEM-H at a concentration of 10^7 cells per 0.5 ml of medium. Cells (10^7) were electroporated at 1,500 V by using a Zapper (U.W. Medical Electronics), resuspended in 10 ml of DMEM-H, and plated in 100-mm-diameter cell culture dishes. Typically 10 μg of DNA was transfected per condition. The pSV2(gpt)-LMP1 and pSV2(gpt) plasmids were used for expression of LMP1 and for relevant controls (60). A total of 2 μg of each pSV2 plasmid was suspended with 8 μg of calf thymus DNA as a carrier, and each suspension (including 10 μg of calf thymus DNA alone) was electroporated into 10^7 cells. Cells were harvested 48 h after transfection directly into RIPA buffer containing SDS loading buffer and analyzed by immunoblotting for EGFR and LMP1 expression as described above.

Tyrosine phosphorylation and immunoprecipitation of the EGFR. C33A derivatives were cultured overnight in serum-free media to minimize background tyrosine phosphorylation due to growth factors in serum. Cells were then incubated for various times in serum-free media supplemented with 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.4) and 500 ng of recombinant EGF (Austral Biologicals) per ml. Cells were lysed directly in RIPA buffer and then diluted into SDS sample buffer. Samples were subsequently analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) for phosphotyrosine-containing proteins as described above. For immunoprecipitation of the EGFR, cells were treated for 2 min with EGF as described above, washed in PBS containing 100 μM Na_3VO_4, lysed in lysis buffer (25 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1% Nonidet P-40, 10 mM NaF, 100 μM Na_3VO_4), and centrifuged for 15 min at 4°C, to pellet cellular debris. The EGFR was precipitated from the resulting supernatant with the anti-ERCT rabbit antiserum, and immune complexes were pelleted with Pansorban (Calbiochem), washed twice in lysis buffer, and analyzed by SDS-PAGE. Immunoblots were analyzed for the EGFR and phosphotyrosine proteins as described above.

RNA isolation and Northern (RNA) blot analysis. Total cellular RNA was isolated by directly lysing cells in guanidine isothiocyanate (GITC), layered over CsCl, and centrifuged for 24 h in an ultracentrifuge. The GITC and CsCl were carefully removed, and the resulting pellet was resuspended in diethyl pyrocarbonate (DEPC)-treated H_2O. Northern blots were prepared with poly(A)$^+$ RNA isolated by using Oligotex-dT beads according to the manufacturer's instructions (Qiagen) or with total cellular RNA. Northern blots were baked in a vacuum oven for 2 h, prehybridized for >2 h (in a buffer containing 6× SSPE [1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA {pH 7.7}], 10× Denhardt's solution, 0.5% SDS, and 50 μg of denatured DNA per ml), and hybridized overnight in the same buffer at $>10^6$ cpm/ml with random primed probes labeled according to the manufacturer's specifications (Promega, Madison, Wis.). The EGFR 64.3, CD23, and β-actin and A20 cDNAs used as probes were kindly provided by J. Schlessinger, E. Kieff, and S. Haskill, respectively.

Reverse transcription-based PCR (RT-PCR) analysis. RNA was extracted as described above from the C33A derivatives, as well as the EGFR-positive HT29 and SiHa cell lines. cDNA was synthesized from 5 μg of total cellular RNA by priming with oligo(dT) and reverse transcribed according to the manufacturer's specifications (Boehringer Mannheim). cDNA corresponding to 0.5 μg of RNA was amplified for 30 cycles in the PCR. Amplifications without reverse transcription were included to ensure amplification of mRNA. Each cDNA preparation was amplified with actin-specific primers to ensure the integrity of the cDNA. EGFR amplification was performed with primers specific for the membrane-bound full-length EGFR (ER10, positions 1813 to 1833, and ER9, positions 2260 to 2280 of the EGFR cDNA) (37). PCR products were subjected to electrophoresis through 1% agarose, and products were visualized by ethidium bromide staining. For analysis of EGFR expression, PCR products were transferred to supported nitrocellulose and filters were hybridized overnight with an end-labeled internal oligonucleotide (ER1, positions 2057 to 2077 of EGFR cDNA) prepared according to the manufacturer's specifications (NEB) (37). In some cases blots were analyzed on a Phosphorimaging screen (Molecular Dynamics).

Stability of EGFR mRNA. mRNA stabilization experiments were carried out by treating 80% confluent cultures with fresh media containing 20 μg of actinomycin D per ml. RNA was isolated at various times posttreatment (0 to 24 h) and analyzed by RT-PCR as described above. Twenty cycles of PCR were performed to keep the PCR at a subplateau level, and gels were transferred to supported nitrocellulose and hybridized with the ER1 probe.

Growth curves of C33A derivatives expressing LMP1. For analysis of the growth characteristics of LMP1-expressing C33A cells and neo controls, cells were trypsinized, pelleted, and washed in serum-free media. Cells were resuspended and plated in duplicate at a density of 5×10^5 cells per tissue culture dish in serum-free media supplemented with 25 ng of EGF per ml. On subsequent days following plating cells were scraped from the dishes and the number of viable cells was assessed by trypan blue exclusion.

RESULTS

Stable expression of LMP1 in human epithelial cells induces EGFR expression. To determine the effect of the EBV-encoded latent membrane protein 1 (LMP1) on the EGFR,

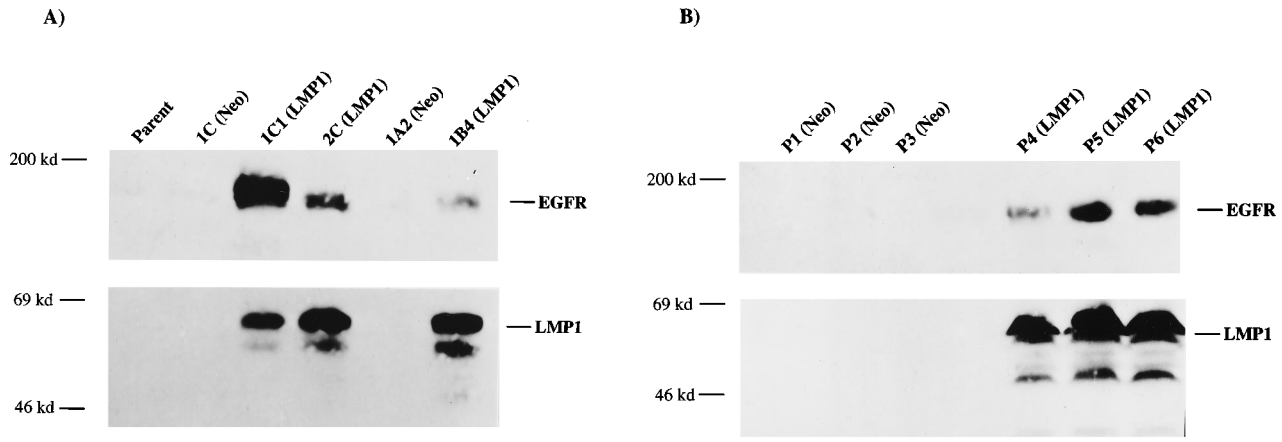

FIG. 1. Induction of EGFR protein in C33A epithelial cells. (A) Immunoblot analysis of C33A subclones transfected with the K14-LMP1 expression construct or the neo control construct. The blots were probed with an anti-EGFR rabbit antiserum or with the anti-LMP1 monoclonal antibody S12. The locations of the 170-kDa EGFR and 63-kDa LMP1 proteins as well as the migration of molecular mass markers are indicated. 1C and 1A2 are subclones which contain the neo control vector, while 1C1, 2C, and 1B4 are LMP1-expressing subclones. Parent represents untransfected C33A cells. (B) Similar analysis of LMP1 and neo cell lines generated by pooling >50 neomycin-resistant colonies. P1 to P3 are neo cell lines, while P4 to P6 are LMP1-expressing cell lines. kd, kilodaltons.

several human epithelial cell lines were transfected with the LMP1 gene under the control of the human keratin 14 (K14) promoter (27). The LMP1-expressing and neo control cell lines derived from the human cervical carcinoma C33A were analyzed by immunoblotting for EGFR (170 kDa) and LMP1 (63 kDa) expression (Fig. 1). Little or no EGFR expression was detected in the parental cell line C33A or in neo control subclones; however, expression of LMP1 caused a dramatic increase in the level of the EGFR protein. Six cell lines expressing LMP1 were analyzed (three subclones and three pools established from >50 colonies), and although there were differences in the expression of both LMP1 and the EGFR between different cell lines, there was a clear correlation between LMP1 expression and expression of the EGFR in all cell lines tested.

In order to further characterize the effect of LMP1 on EGFR expression, two additional cell lines, HT29 colonic carcinoma cells and ME-180 cervical carcinoma cells were studied. Both of these cell lines express relatively high levels of the EGFR which were unaffected by expression of LMP1 (data not shown). These experiments indicate that LMP1 is able to induce expression of the EGFR in human epithelial cells lacking detectable EGFR but that it did not significantly alter EGFR expression in several cell lines expressing the EGFR.

Induction of the EGFR by LMP1 in a transient assay. LMP1 has been shown to exert toxic effects when expressed at high levels in several cell types (1, 23, 39). To eliminate the possibility that overexpression of the EGFR resulted from an indirect mechanism to overcome the toxic effect of LMP1 in established cell lines, the effect of LMP1 on EGFR expression was determined in a transient assay. C33A cells were transfected with the pSV2(gpt) and pSV2(gpt)-LMP1 vectors, which contain the human metallothionein promoter and capping site driving the expression of LMP1 (60). Cells were harvested 48 h after transfection and analyzed by immunoblotting for LMP1 and EGFR expression (Fig. 2). As in the previous experiments with LMP1-derived cell lines, both the parental cells and cells transfected with the control pSV2(gpt) vector exhibited little or no EGFR expression. In contrast, cells transfected with the pSV2(gpt)-LMP1 vector expressed considerable levels of both the LMP1 and EGFR gene products. These results confirm the previous finding that expression of LMP1 was directly responsible for the increased EGFR expression and that this increased expression was not due to secondary changes occurring in long-term cultures brought about by LMP1 expression.

EGFR expression in EBV-infected lymphocytes. Since EBV infects both epithelial cells and B lymphocytes in vivo, the effect of LMP1 expression on the EGFR was investigated in B-lymphocyte cell lines. Expression of the EGFR is normally restricted to cells of epithelial and mesenchymal origin, and this expression is not normally detected in hematopoietic cells,

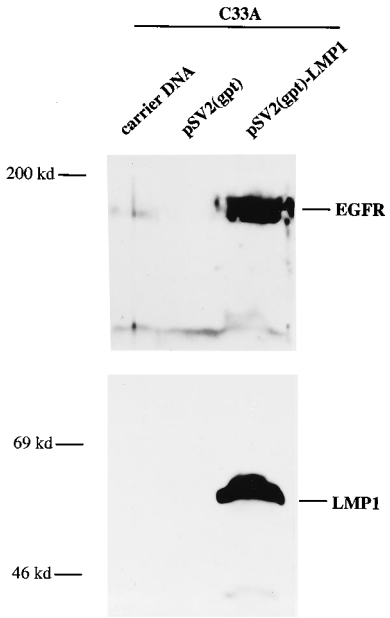

FIG. 2. Induction of EGFR expression through transient expression of LMP1 in C33A cells. Cells (10^7) were transfected with 2 μg of either the LMP1 expression plasmid pSV2(gpt)-LMP1 or the control pSV2(gpt) plasmid along with 8 μg of carrier DNA. As an additional control 10 μg of carrier DNA alone was transfected. At 48 h after transfection, cells were harvested and analyzed by immunoblot analysis for the 170-kDa EGFR and 63-kDa LMP1 proteins as described in the legend to Fig. 1. kd, kilodaltons.

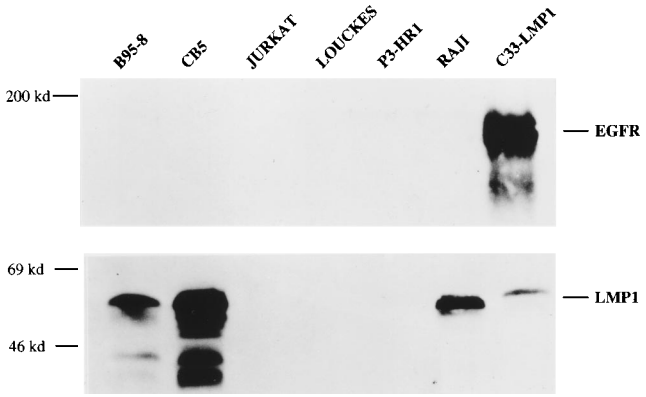

FIG. 3. LMP1 expression in B lymphocytes does not alter EGFR expression. Extracts from 5×10^5 cells were analyzed for EGFR and LMP1 expression as described in the legend to Fig. 1. B95-8 is an EBV-infected marmoset cell line, and CB5 is a human lymphoblastoid cell line established with B95-8 virus. Jurkat and Louckes are EBV-negative T- and B-cell lymphomas, respectively. P3-HR-1 is a subclone of a BL cell line which does not express LMP1. Raji is an LMP1-expressing BL cell line. C33-LMP1 is a subclone of C33A epithelial cells that expresses LMP1. The locations of the 170-kDa EGFR and 63-kDa LMP1 proteins as well as the migration of molecular mass markers are indicated. kd, kilodaltons.

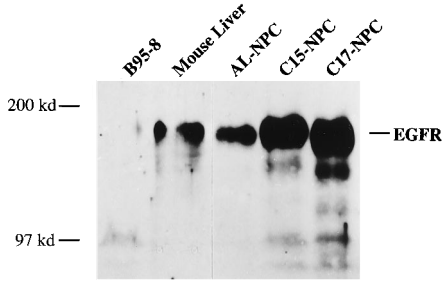

FIG. 4. LMP1 expression in NPC tumor samples. Immunoblots containing 100 μg of membrane proteins extracted from NPC samples were analyzed for EGFR expression as described in the legend to Fig. 1. B95-8 is an EBV-positive marmoset B-cell line. Mouse liver is normal mouse liver tissue obtained at biopsy from a mouse harboring a C15 tumor. AL is an NPC metastasis of the liver and was obtained at autopsy. C15 and C17 are nude-mouse-passaged NPC tumors. kd, kilodaltons.

including B lymphocytes. Although the EGFR could be readily detected in the C33A cells expressing LMP1, expression of the EGFR was not detected by immunoblot analysis in any of the lymphocyte cell lines (Fig. 3). The cell lines examined included the EBV-infected marmoset and human lymphoid cell lines B95-8 and CB5 and the BL cell line Raji. These cell lines all express significant levels of the LMP1 protein. Additionally, EGFR expression was not detected in the EBV-infected, defective BL cell line P3-HR-1, the EBV-negative BL Louckes cell line, or the T-cell leukemia Jurkat cell line. Thus, expression of the EGFR is not likely to be important in lymphomagenesis, regardless of the status of EBV. These results also indicated that expression of LMP1 has distinct effects on cellular gene expression in epithelial and lymphoid cells.

Expression of the EGFR in EBV-infected NPC tissue. The in vitro experiments indicated that the EBV-encoded LMP1 gene is capable of inducing expression of the EGFR in established human epithelial cells. EBV is strongly associated with the human epithelial cell malignancy NPC. Thus, it is likely that LMP1 is an important factor in the events leading to tumor formation. The expression of the EGFR in several NPC tumors, including the C15 and C17 tumors passaged in nude mice, as well as an NPC sample (AL) which was obtained at autopsy, was examined. Crude membrane fractions were prepared from each tumor, and 100 μg of protein from each sample was subjected to SDS-PAGE with equal loadings demonstrated by Coomassie blue staining (data not shown). Immunoblot analysis using the EGFR-specific anti-ERCT rabbit antiserum demonstrated high-level expression of the EGFR in the two nude-mouse-passaged NPC tumors compared with control mouse liver tissue (Fig. 4). The AL sample contained intermediate levels of the EGFR, which could be an underestimate of the total EGFR level in this tumor since NPC samples normally contain significant levels of infiltrating lymphocytes not found in nude-mouse-passaged NPC samples. Expression of LMP1 was detected in each of the NPC samples analyzed (14, 50, 51, 64). The upregulation of the EGFR in human epithelial cell lines in vitro by LMP1 and the expression of the EGFR in NPC suggest that LMP1 may contribute to the development of NPC in part through induction of the EGFR.

Ligand-dependent activation of EGFR tyrosine kinase in LMP1-expressing C33A cells. Stimulation of cell lines expressing the EGFR with EGF normally results in a rapid and transient activation of the EGFR tyrosine kinase. In order to determine if the EGFR upregulated by LMP1 in C33A cells is a functional tyrosine kinase, LMP1-converted C33A cells and neo control C33A cells were treated with 500 ng of EGF per ml for 2 min and the EGFR was immunoprecipitated with the anti-ERCT antiserum. Immunoprecipitates were subjected to SDS-PAGE and analyzed by immunoblotting for either total EGFR (Fig. 5A, anti-ERCT) or tyrosine-phosphorylated EGFR by using anti-phosphotyrosine antibodies (Fig. 5B, anti-PY). As expected, the EGFR was detected only in the C33A cells expressing LMP1. The anti-ERCT antibody detects both phosphorylated and unphosphorylated EGFR; therefore, the EGFR was detected in immunoprecipitates from treated and untreated cells (Fig. 5A). Upon treatment with EGF, the EGFR in the LMP1-expressing cells was phosphorylated on tyrosine as evidenced by its reactivity with the anti-phosphotyrosine antibody (Fig. 5B). Tyrosine phosphorylation was not detected in the neo control C33A cells, or in the untreated LMP1-expressing C33A cells. These data indicate that the EGFR upregulated by LMP1 is functional and capable of stimulation by the mitogen EGF.

Induction of EGFR tyrosine kinase activity is known to initiate tyrosine phosphorylation of several substrate proteins which ultimately lead to altered gene expression and DNA synthesis. To determine the pattern of tyrosine-phosphorylated proteins in C33A epithelial cells, neo control and LMP1-expressing cells were stimulated with EGF and whole-cell lysates were subjected to immunoblot analysis with anti-phosphotyrosine antibodies (Fig. 5C). Cells were treated with EGF and harvested at various time points following stimulation. As expected, the 170-kDa EGFR was rapidly phosphorylated on tyrosine in cells expressing LMP1, while phosphorylation was not detected in the neo control cells. Additional proteins, including pp120, pp85, pp75, and pp55, were also phosphorylated on tyrosine in the LMP1 expressing cells. Phosphorylation of these additional proteins was dependent both on EGF and on the EGFR, as phosphorylation of these proteins was not detected in the non-EGFR-expressing neo control cells treated with EGF. Tyrosine phosphorylation in the LMP1-expressing cells was analyzed at later time points to determine the time course of dephosphorylation. The EGFR and substrate proteins remained tyrosine phosphorylated up to 30 min following stimulation with EGF, although the levels of phosphorylation gradually decreased with time (Fig. 5C). Examina-

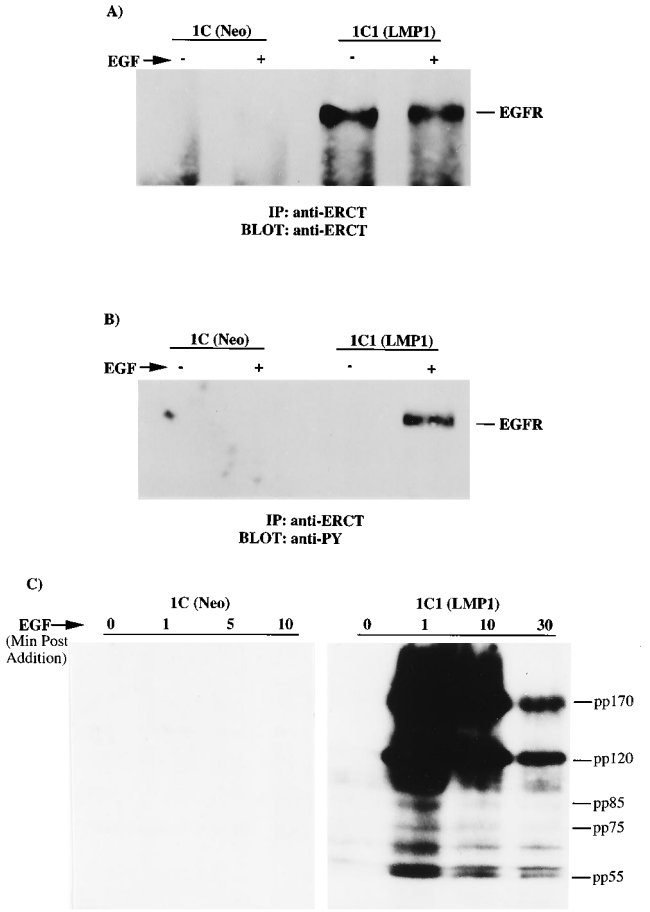

FIG. 5. Phosphotyrosine analysis of the EGFR and substrate proteins in LMP1-converted C33A cells. LMP1 and control C33A cells were treated with EGF for 2 min, and the EGFR was immunoprecipitated as described in Materials and Methods. Immunoprecipitates were analyzed by immunoblotting for total EGFR with the anti-ERCT rabbit antiserum (A) or for tyrosine-phosphorylated EGFR with the anti-phosphotyrosine (anti-PY) antibody PT-66 (B). (C) Tyrosine phosphorylation in whole-cell lysates as analyzed by immunoblotting with the anti-PY antibody PT-66. LMP1-expressing and neo control cells were treated with EGF for various times and analyzed for phosphotyrosine-containing proteins. The blot is intentionally overexposed to visualize less-prominent bands in the range of 55 to 85 kDa.

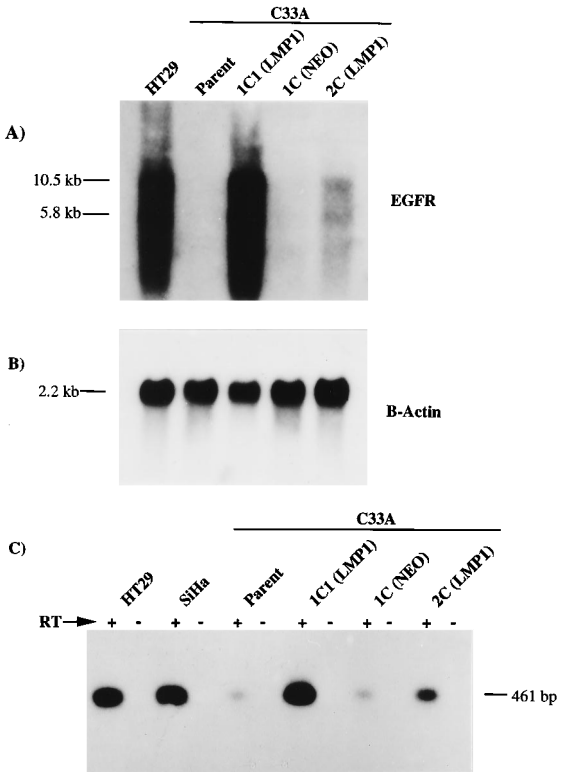

FIG. 6. Expression of EGFR-specific mRNAs in C33A cells expressing LMP1. (A) Poly(A)$^+$ RNA (15 μg) isolated from LMP1-expressing and control subclones analyzed by Northern blotting for EGFR expression with the 64.3 EGFR cDNA probe. The 10.5- and 5.8-kb mRNA species are indicated. An equal amount of RNA from the EGFR-expressing HT29 cell line was included as a control. (B) The blot was reprobed with a β-actin cDNA to demonstrate equal loading and integrity of the RNAs. (C) RT-PCR analysis of EGFR mRNA in the C33A subclones. cDNA corresponding to 0.5 μg of RNA was amplified for 20 cycles of PCR as described in Materials and Methods. cDNA synthesis without reverse transcription was performed to ensure amplification of RNA. After electrophoresis through 1% agarose gels, PCR products were transferred to nitrocellulose and probed with ^{32}P-labeled ER1 oligonucleotide as described in Materials and Methods. Equal amounts of cDNA from the EGFR-positive HT29 and SiHa cell lines were included as controls.

tion of tyrosine phosphorylation patterns in LMP1-expressing ME-180 cells, which express the EGFR in the absence of LMP1, revealed similar levels of tyrosine-phosphorylated proteins whose dephosphorylation exhibited a time course similar to that of proteins in the neo control ME-180 cells (data not shown). These data indicate that LMP1 does not significantly alter the rate of downregulation by affecting internalization, dephosphorylation, or degradation of the EGFR.

Induction of EGFR mRNA levels by LMP1. To determine if the increased level of EGFR protein detected in LMP1-expressing C33A cells was due to an increased mRNA level, the expression of the EGFR mRNA was analyzed by Northern blotting. Samples of poly(A)$^+$ RNA from LMP1-expressing cells, neo controls, and C33A parental cells were hybridized with the 64.3 cDNA probe encompassing 768 bp of the EGFR open reading frame 3′ to the putative transmembrane domain (57). The HT29 cell line, which expresses the EGFR, was included as a positive control. Hybridization was not detected in the parental C33A cells, while mRNAs of 10.5 and 5.8 kb were detected in the LMP1-expressing cells and the HT29 cells with the 64.3 cDNA probe (Fig. 6A). The 10.5- and 5.8-kb mRNA species were also detected in RNA preparations from the C15 NPC tumor, which expresses the EGFR protein, and were identical in size to the mRNAs detected in other cell types expressing the EGFR (Fig. 4 and data not shown) (11, 57). The 10.5- and 5.8-kb EGFR mRNAs both initiate in the same promoter region and differ in the 3′ untranslated region (31). The Northern blot was reprobed with a cDNA probe for β-actin to ensure equal loading of the mRNAs (Fig. 6B).

Expression of the EGFR was also analyzed by RT-PCR (Fig. 6C). Amplifications were also performed on equivalent amounts of cDNA from the EGFR-expressing HT29 and SiHa epithelial cell lines. As expected, the LMP1-expressing subclones 1C1 and 2C contained elevated levels of EGFR transcripts compared with the C33A parental cells and the 1C neo control subclone. The 1C1 subclone expressed more EGFR RNA than the 2C subclone, consistent with previous analysis of EGFR levels as detected by Northern blot analysis of mRNA and Western blot (immunoblot) analysis of total cellular protein. Low levels of EGFR transcription were detected by RT-PCR in the parental cells and neo subclone 1C. These data confirm the finding that LMP1 expression elevates EGFR mRNA levels.

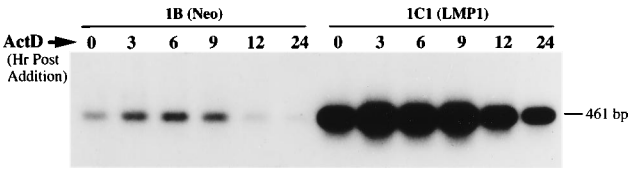

FIG. 7. Stability of the EGFR mRNA. C33A cells expressing LMP1 and control cells were treated at 80% confluence with 20 μg of actinomycin D, and RNA was isolated at various time points following treatment. cDNA was prepared as described in Materials and Methods. After electrophoresis and transfer to nitrocellulose, the blot was probed with ^{32}P-labeled ER1 oligonucleotide and analyzed with a Phosphorimaging screen (Molecular Dynamics).

Stability of EGFR mRNA. In order to more precisely determine the mechanism leading to induction of steady-state levels of EGFR mRNA, the effect of LMP1 expression on the stability of the EGFR message was investigated. Previous reports have suggested that the accumulation of EGFR mRNA expression following EGF stimulation involved stabilization of the EGFR message (28). C33A cells expressing LMP1 (1C1) and the vector control (1C) were treated with 20 μg of actinomycin D per ml at 80% confluence for 0 to 24 h, and RNA was harvested at several times after treatment. Since EGFR mRNA levels could be detected in the neo control cells only by RNA PCR, this methodology was utilized to determine the turnover of the EGFR mRNA in the two subclones. Determination of EGFR mRNA turnover in the 1C1 (LMP1 expressing) subclone by Northern and RNA PCR analysis gave similar results, indicating that the RNA PCR analysis would be valid in a determination of EGFR mRNA turnover (data not shown). cDNA was subjected to 20 rounds of PCR amplification as described above and hybridized with an internal oligonucleotide corresponding to the EGFR sequence (Fig. 7). Quantitation of the EGFR mRNA on a Phosphorimaging screen (Molecular Dynamics) indicated that the half-life of the mRNA was approximately 12 h in both the LMP1-expressing and control subclones of C33A cells.

Analysis of the short-lived c-Fos mRNA levels demonstrated that c-Fos mRNA was completely degraded by 3 h, confirming the shutoff of transcription by actinomycin D in these cells (data not shown). Previous analyses have indicated that the half-life of the EGFR mRNA in epithelial cells is approximately 3 h, while the data presented here suggest that the half-life of this mRNA may be significantly longer in some epithelial cells (21, 28). These data indicate that LMP1 does not alter the half-life of the EGFR message and suggest that induction of EGFR transcription leads to the increased steady-state levels of EGFR mRNA.

Expression of LMP1 in C33A epithelial cells induces A20 expression. LMP1 expression in B lymphocytes induces a number of cellular changes, including the upregulation of mRNA levels of A20, bcl-2, and CD23 (FcεRIIb form) (24, 34, 61). Induction of gene expression by LMP1 in lymphocytes is thought to be responsible for cellular activation as well as prevention of apoptosis. To investigate the expression of A20 in the C33A epithelial cells expressing LMP1, Northern blots of total cellular RNA were hybridized with a cDNA probe specific for the A20 gene (Fig. 8). C33A cells expressing LMP1, P4 (LMP1) and P5 (LMP1), also expressed significant levels of A20 mRNA, while the neo control cells, P1 (Neo) and P2 (Neo), did not have detectable levels of A20 mRNA. In, contrast CD23 and bcl-2 gene expression was not altered in the C33A cells expressing LMP1 (data not shown). These data indicate that LMP1 expression has differential effects on lymphocytes and epithelial cells, with A20 upregulation being common to both cell types, CD23 and bcl-2 upregulation being specific to lymphocytes, and EGFR upregulation being specific to epithelial cells.

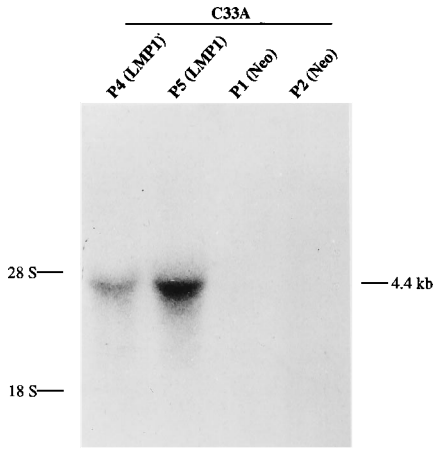

FIG. 8. Expression of A20-specific mRNAs in C33A cells expressing LMP1. Aliquots of RNA (40 μg) were isolated from LMP1-expressing (P4 and P5) and neo control (P1 and P2) C33A cell lines and analyzed by Northern blotting with the A20 cDNA probe. The locations of rRNAs and the 4.4-kb A20 mRNA are indicated.

LMP1 expression alters the growth rates of C33A cells in serum-free media. Single gene transfer experiments with the EGFR have demonstrated that the EGFR can transform rodent fibroblasts in vitro in a ligand-dependent manner (58). The growth pattern of LMP1-expressing C33A cells was compared with that of neo control C33A cells in serum-free media supplemented with 25 ng of EGF (Austral Biologicals) per ml. Cells (5×10^5) were seeded in fresh media, and viable cells (on the basis of trypan blue exclusion) were counted at intervals following plating in the serum-free media (Fig. 9). The data presented are the results of three independent experiments

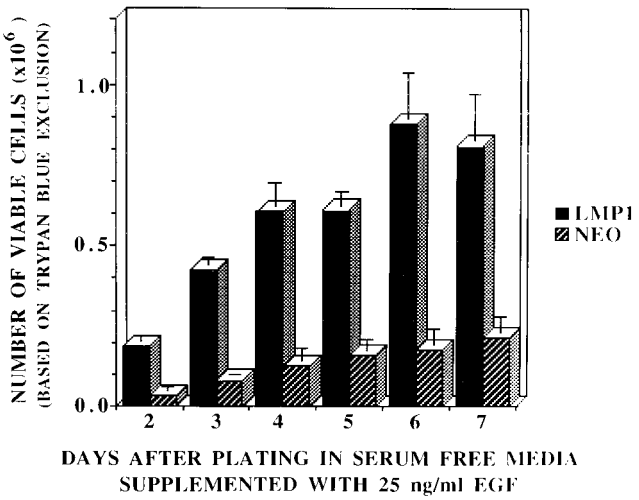

FIG. 9. Growth of LMP1-expressing C33A cells in serum-free media supplemented with EGF. Exponentially growing C33A LMP1-expressing cells and neo control cells were harvested, washed once in serum-free media and seeded at a density of 5×10^5 cells per plate in serum-free media supplemented with 25 ng of recombinant EGF (Austral Biologicals) per ml. On various days after seeding, the cells were harvested and cell viability was assessed on the basis of trypan blue exclusion. The numbers and errors depicted in the graph represent three individual experiments performed in duplicate.

performed in duplicate. After an initial decrease in cell number, probably due to plating efficiency of the C33A cells in serum-free media, the C33A cells expressing LMP1 increased in numbers to much greater densities than the neo controls. At day 6 following plating, the LMP1-expressing C33A cells had increased in number to 8.8×10^6 cells per plate, while the neo control cells had increased to only 1.8×10^6 cells per plate. Following day 6, both populations of cells began to decrease in viability, indicating that while short-term cultures of LMP1-expressing C33A cells were able to grow in serum-free media, LMP1 was unable to transform C33A cells to complete serum independence. These data suggest that, in a manner similar to that of its effects in lymphocytes, LMP1 induces alterations in epithelial cells that influence cellular growth and differentiation.

DISCUSSION

The EBV-encoded LMP1 protein is one of several proteins essential for growth transformation of B lymphocytes in vitro, and it is the only EBV-encoded protein shown to transform rodent fibroblasts (1, 30, 60). The protein is expressed in EBV-immortalized B lymphocytes and has also been detected in approximately 65% of all NPC tumors (14, 64). The lack of a suitable in vitro infection system has slowed the understanding of the potential role of LMP1 in these tumors; however, several studies using single gene transfer of LMP1 into epithelial cells revealed that LMP1 affects epithelial cell growth and differentiation. In the SCC12F squamous carcinoma cell line, expression of LMP1 inhibited terminal differentiation of polarized cells grown on feeder layers, while LMP1 expression in the immortalized RHEK keratinocyte cell line induced tumorigenicity in nude mice (9, 15). The underlying molecular events that affect cellular growth and differentiation by LMP1 in epithelial cells are unknown.

The EGFR is a membrane-bound tyrosine kinase frequently overexpressed in epithelial tumors (62). Ligand-dependent activation of the EGFR has been shown to transform rodent fibroblasts in vitro; however, the mechanisms leading to the deregulation of EGFR in human tumors are not well understood (31, 58). Increased levels of EGFR protein and mRNA have been observed and amplification of the gene encoding the EGFR has been detected in a number of cell lines derived from epithelial tumors (33, 62). Regulation of EGFR expression, EGFR kinase activity, and the expression of aberrant EGF receptors have also been shown to be associated with viral oncogenesis. The avian erythroblastosis retrovirus encodes a truncated EGFR protein consisting of the transmembrane and tyrosine kinase domains of the cellular EGFR (13). This truncated protein possesses a constitutively active tyrosine kinase and efficiently transforms chicken embryo fibroblasts in culture (16, 19). The E5 open reading frame of both bovine papillomavirus and human papillomavirus prolongs the stimulated tyrosine kinase activity of the EGFR, possibly because of inhibition of EGFR degradation. This results in increased levels of activated EGFR on the cell surface (35, 55). The hepatitis B virus X-gene product increases EGFR levels when expressed in human hepatoma-derived cells through upregulation of the EGFR promoter, resulting in increased steady-state levels of both EGFR mRNA and protein (36). These studies suggest that altered regulation of the EGFR signal, either by amplification or overexpression or in experimental models through the action of viral oncogenes, may be an important step in the transformation of epithelial cells. The data presented here reveal that the EBV-encoded LMP1 protein also targets the EGFR in epithelial cells by upregulating the steady-state expression of EGFR mRNA and protein.

Overexpression of the EGFR has also been shown to alter the abilities of cell lines to form tumors in nude mice. Introduction of a human EGFR expression construct into NIH 3T3 cells rendered the cells capable of forming tumors in nude mice (58). In addition, alteration of the EGFR number in MCF-7 and A431 human epithelial cell lines altered their tumor forming capacity (53, 59). The MCF-7 breast epithelial cell line, which expresses low levels of the EGFR, forms tumors in vivo only in an estrogen-dependent manner. However, selection of adriamycin-resistant MCF-7 cell lines, which overexpress the EGFR, allowed hormone-independent tumor formation (59). Selection of A431 cells with decreased levels of EGFR reduced tumor volume and slowed tumor growth, suggesting the importance of EGFR overexpression in the formation of tumors in vivo (53). In this study, the accumulation of the EGFR protein enabled the cells to grow to higher cell densities when plated in serum-free media supplemented with EGF. This suggests that LMP1-induced EGFR overexpression influences cellular growth. The finding that the EGFR is also overexpressed in NPC tissue suggests that upregulation of EGFR expression may contribute to the development of NPC.

Analyses of tyrosine phosphorylation indicated that the LMP1-induced EGFR is fully functional and is capable of binding to EGF and becoming phosphorylated on tyrosine residues. The additional proteins, including pp55, pp75, pp85, and pp120, that became tyrosine phosphorylated after treatment with EGF are similar to those detected in other epithelial cells following stimulation of the EGFR with EGF (6, 10). Previous studies of the EGFR suggest that the 55-kDa phosphoprotein in the EGF-stimulated C33A cells may be the Shc protein. Shc has been shown to link the EGFR to the Ras pathway through interaction with Grb2 (2, 45, 49). Since it has been suggested that LMP1 can mimic activated Ras in the prevention of terminal differentiation of stratified epithelial cell cultures, it is possible that LMP1 exerts this physiologic effect in part by upregulating expression of the EGFR (9). Activation of the EGFR in some cells leads to tyrosine phosphorylation of the 85-kDa regulatory subunit of the phosphatidylinositol 3′-kinase (PI3-kinase). In this manner the EGFR activates the phosphoinositol second messenger pathway (5, 26, 54). Interestingly, the interaction of polyomavirus middle T antigen with phosphatidylinositol 3′-kinase may be involved in the transformation of 3T3 cells, as mutants of middle T that failed to interact with phosphatidylinositol 3′-kinase failed to transform (5). Tyrosine phosphorylation of substrate proteins in LMP1-transfected C33A cells suggests that activation of signal transduction cascades resulting from LMP1 expression may be important in epithelial cell proliferation and transformation. It will be particularly important to determine whether LMP1 also enhances production of an EGFR ligand such as transforming growth factor α, thereby setting up a self-stimulatory autocrine loop.

The analyses of EGFR expression indicate that the EGFR induced by LMP1 is due to an increase in the steady-state level of EGFR mRNA. Overexpression of EGFR mRNA in human tumor cell lines has been shown to involve mRNA stabilization, promoter activation, and relief of transcription termination signals in intron 1 of the EGFR gene (7, 11, 20, 21, 28). The stability of the EGFR mRNA was not affected by LMP1, suggesting that LMP1 activates EGFR transcription. Experiments with lymphocytes have indicated that LMP1 may at least in part activate transcription through induction of the NF-κB transcription factor (22, 34, 48). The EGFR promoter sequences that have been previously analyzed contain 1.3 kb 5′ to

the transcription initiation site and do not contain any identifiable NF-κB binding sites (29). The activity of the EGFR promoter-CAT recorder constructs in C33A and ME-180 cells was discordant with the level of endogenous EGFR expression, and the promoter constructs were not activated by LMP1 (data not shown). Previous studies have suggested the presence of important regulatory sequences in intron 1 of the EGFR gene. The direct induction of EGFR mRNA by LMP1 will enable the identification of important regulatory sequences for EGFR transcription and potentially lead to the identification of additional transcriptional pathways that are activated by LMP1.

As in B lymphocytes, LMP1 upregulated expression of the A20 gene in the C33A epithelial cells. The A20 promoter has been cloned and was shown to be induced through the activation of NF-κB by LMP1 (34). Expression of LMP1 in C33A epithelial cells does activate NF-κB family members, and so it is likely that upregulation of A20 gene expression in C33A cells is a result of NF-κB activation (43). The expression of other NF-κB-inducible proteins and their influence on epithelial cell growth are likely to be important in the formation of NPC.

A20 expression has been shown to protect cells from tumor necrosis factor alpha induced apoptosis (42). Interestingly, LMP1 has recently been shown to engage signaling proteins for the tumor necrosis factor family receptors (40). Activation of these signaling molecules by LMP1 could mimic ligand binding to this family of receptors, and therefore subsequent induction of A20 may be important for the protection of apoptosis induced by activation of this pathway.

This is the first identification of upregulation of a cellular tyrosine kinase by the EBV-encoded LMP1 gene. The EGFR induced by LMP1 is a fully functional tyrosine kinase that appears to activate signal transduction cascades important in cellular proliferation. In addition, increased growth of LMP1-converted C33A cells in serum-free media may reflect the enhanced proliferation effected by the EGFR and possibly the inhibition of apoptosis by the A20 molecules. The upregulation of a biologically active EGFR molecule may represent an important contribution by LMP1 to the formation of epithelial-cell-derived tumors like NPC.

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