

## Epstein-Barr Virus LMP1 Induction of the Epidermal Growth Factor Receptor Is Mediated through a TRAF Signaling Pathway Distinct from NF- $\kappa$ B Activation

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**The Epstein-Barr virus (EBV)-encoded LMP1 protein induces several cellular changes including induction of epidermal growth factor receptor (EGFR) expression and activation of the NF- $\kappa$ B transcription factor. Two domains within the carboxy terminus have been identified that activate NF- $\kappa$ B. In this study, mutational analysis of the LMP1 protein indicated that the proximal NF- $\kappa$ B activation domain, which is identical to the TRAF interaction domain (amino acids 187 to 231), is essential for induction of the EGFR. The distal NF- $\kappa$ B activation domain (amino acids 352 to 386) did not induce expression of the EGFR. In contrast, the two domains both independently activated a  $\kappa$ B-CAT reporter gene and induced expression of the NF- $\kappa$ B-regulated A20 gene in C33A epithelial cells. These results indicate that induction of the EGFR by LMP1 involves the TRAF interaction domain and that activation of NF- $\kappa$ B alone is not sufficient. Northern blot analysis revealed that induction of EGFR and A20 expression is likely to be at the transcriptional level. Interestingly expression of CD40 in the C33A cells also induced expression of the EGFR. Overexpression of either TRAF3 or an amino-terminal-truncated form of TRAF3 (TRAF3-C) inhibited signaling from the LMP1 TRAF interaction domain but did not affect signaling from the distal NF- $\kappa$ B activation domain. These data further define the mechanism by which LMP1 induces expression of the EGFR and indicate that TRAF signaling from LMP1 and CD40 activates a downstream transcription pathway distinct from NF- $\kappa$ B that induces expression of the EGFR.**

The Epstein-Barr virus (EBV), a ubiquitous human gamma-herpesvirus, is consistently associated with several human malignancies including endemic Burkitt's lymphoma, Hodgkin's disease, posttransplant lymphoma, and nasopharyngeal carcinoma (NPC) (30, 45, 47, 66). Infection of primary B lymphocytes in vitro with EBV leads to a restricted array of EBV gene expression and subsequent immortalization of cells into continuous lymphoblastoid cell lines. Genetic analyses have indicated that expression of latent membrane protein 1 (LMP1) and five nuclear antigens (EBNA1, -2, -3A, -3C, and -LP) are critical for the immortalization of primary B lymphocytes (7, 29, 35, 60). Expression of latent genes that are not essential for B-cell immortalization in vitro, such as EBNA3B, LMP2, and the EBV-encoded small RNAs, are likely to have a role in EBV infection in vivo (33, 36, 37, 59).

The function of EBV gene products expressed in epithelial malignancies such as NPC is much less understood due to the lack of an in vitro infection and transformation system. In primary NPC biopsies and NPCs passaged in nude mice, EBV gene expression is more restricted than that in latently infected lymphocytes, with expression limited to LMP1, LMP2, EBNA1, and a series of transcripts from the *Bam*HI A region of the genome (5, 14, 15, 20, 50). A recent study of rare preinvasive NPC lesions has demonstrated LMP1 expression in all cells in 100% of cases, suggesting an important role for this protein in the development of malignancy (46).

Expression of LMP1 in B-lymphoma cell lines induces many of the phenotypic changes characteristic of EBV immortalization of primary lymphocytes. LMP1 induces the expression of the B-cell activation antigens CD23 and CD40, adhesion mol-

ecules such as ICAM-1, LFA-1, and LFA-3, as well as the CaM kinase-Gr and a novel p40 interleukin (IL)-12-related protein (10, 42, 64, 65). LMP1 also induces the expression of several molecules that function as inhibitors of the cell death pathway such as bcl-2 and A20 (18, 31, 43). The activation of the NF- $\kappa$ B transcription factor by LMP1 is likely to mediate many but probably not all of the changes in gene expression that are affected by LMP1 (17, 24, 39, 44).

LMP1 can also induce cellular changes in cell types other than lymphocytes and is the only EBV protein capable of transforming rodent fibroblasts in vitro (1, 40, 62). Recently LMP1 has been shown to induce expression of the epidermal growth factor receptor (EGFR) and A20 molecule in human epithelial cells (38). Upon stimulation with EGF, these cells demonstrate enhanced tyrosine phosphorylation of downstream targets of the EGFR and exhibit enhanced growth in serum-free media. Induction of molecules such as the EGFR and A20 in epithelial cells may be important for LMP1-mediated effects on cell growth and differentiation in established epithelial cell lines, as well as for EBV-infected epithelial malignancies such as NPC.

The tumor necrosis factor receptor (TNFR) family comprises a large group of transmembrane proteins that transmit extracellular signals but do not contain the intrinsic tyrosine kinase activity typically associated with membrane receptors (55, 58, 61). Studies have indicated that activation of the TNFR family member CD40, in conjunction with IL-4 stimulation, can mimic EBV-induced immortalization of human lymphocytes in short-term culture (2, 13, 51). Expression of LMP1 in B-lymphoid cells induces many of the phenotypic changes characteristic of EBV immortalization, suggesting parallel roles for the cellular CD40 protein and the viral LMP1 protein in signal transduction pathways. The CD40 protein is

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expressed on the cell membrane of several cell types other than activated B cells. CD40 expression has been detected in carcinomas but not normal epithelial cells and also in EBV-infected NPC tissue (4, 56, 67). The expression of CD40 and LMP1 on malignant epithelial cells suggests that signals from these proteins may be involved in not only B-cell activation but also progression to malignancy in cells of epithelial origin such as NPC.

The recent identification of putative signaling proteins called TNFR associated factors (TRAFs) has provided a new understanding into the function of molecules like LMP1 and CD40 (49). The carboxy terminus of LMP1 (amino acids 187 to 386) has been shown to interact with TRAF1, TRAF2, and TRAF3 (9, 41). More precise mapping indicated that there is a single TRAF interaction domain located within amino acids 187 and 231 of LMP1 (9, 41). TRAF3 was also cloned in the yeast two-hybrid system by its ability to interact with the cytoplasmic domain of the CD40 protein (6, 22, 52).

Two effector domains have been identified within the LMP1 cytoplasmic C terminus which mediate activation of NF- $\kappa$ B (24, 39). The proximal domain (amino acids 187 to 231) is identical to the TRAF interaction domain and is a weaker activator of NF- $\kappa$ B than the distal domain (amino acids 352 to 386) (24, 39). Recombinant EBV molecular genetic analysis of LMP1 has indicated that the TRAF interaction domain is important for B-lymphocyte transformation while the distal domain is dispensable (28). The present study was designed to identify the domains of LMP1 that are essential for induction of EGFR expression and to determine if this induction involves TRAF signaling and/or NF- $\kappa$ B activation.

#### MATERIALS AND METHODS

**Cell lines and establishment of derivatives.** C33A epithelial cells, derived from a human cervical carcinoma, were grown at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Gibco BRL) and antibiotics. ME-180 epithelial cells, also derived from a cervical carcinoma, were grown as described above except they were grown in McCoy's 5A medium. Cells were routinely grown in 100-mm-diameter cell culture dishes and subcultured three times weekly. EBV-immortalized lymphocytes (CB5) transformed by the EBV B95-8 strain were maintained at between  $2 \times 10^5$  and  $1 \times 10^6$  cells per ml in RPMI 1640 supplemented with 10% fetal calf serum and antibiotics. C33A cell lines expressing CD40, LMP1, and mutants of LMP1, were obtained by selection in 600  $\mu$ g of G418 (Gibco-BRL) per ml added 48 h after transfection as described below. In order to avoid clonal variation in individual cells, each cell line was generated by pooling  $>50$  G418-resistant colonies.

**Plasmids and expression vectors.** The plasmid containing the CD40 cDNA was kindly provided by Hitoshi Kikutani. The expression vector used for most of the constructs in this study is the pcDNA3 vector (Invitrogen) which contains the cytomegalovirus (CMV) immediate-early promoter and polyadenylation sequence from the bovine growth hormone gene. The LMP1 cDNA was subcloned into the *EcoRI* site of pcDNA3. The LMP1 gene was also subcloned into a pGEM2-based vector containing the keratin-14 promoter (K14) (38). The CD40 cDNA was subcloned into the *HindIII* and *XhoI* sites of pcDNA3. Expression vectors for full-length TRAF3 and amino-terminally truncated (amino acids 347 to 568) TRAF3-C proteins were created by subcloning the cDNAs into pSG5. The pSG5 vector contains the simian virus 40 early promoter and intron sequences from the rabbit  $\beta$ -globin gene (Stratagene). The reporter construct,  $\kappa$ B-CAT, contains two copies of the  $\kappa$ B site from the human immunodeficiency virus-long terminal repeat (HIV-LTR) (-105 to -75) cloned upstream of the TATA region from the *Xenopus* albumin promoter linked to the chloramphenicol acetyltransferase (CAT) gene (54, 57). TATA-CAT is identical to  $\kappa$ B-CAT except that it does not contain the tandem copies of the  $\kappa$ B sites derived from the HIV-LTR.

**Construction of LMP1 mutants.** All the LMP1 mutants were cloned into the pcDNA3 expression vector. The DEL 1-128 mutant (amino acids 129 to 386) contains the *XhoI/EcoRI* fragment of LMP1 cloned into the *BamHI* and *EcoRI* sites of pcDNA3. Initiation of translation would begin at the methionine located at amino acid 129 of the wild-type LMP1 protein. All the other LMP1 mutants were cloned into the *EcoRI* site of pcDNA3. The DEL 187-351 mutant was constructed by removal of the 495-bp *NcoI* restriction fragment in the LMP1 cDNA, followed by recircularization of the resultant plasmid. The 187-STOP and 231-STOP mutants were constructed by blunt-end ligation of a stop codon containing double-stranded oligonucleotide (5'-CTAGTCTAGACTAG-3'). The 187-STOP mutant ( $\Delta$ 188-386) was made by cloning the nonsense oligonucleotide

into the Klenow end-filled *NcoI* site in the LMP1 cDNA. The 231-STOP ( $\Delta$ 232-386) mutant was made by cloning the nonsense oligonucleotide into the *NaeI* site in the LMP1 cDNA. FLAG-tagged LMP1 (DEL 187-351 and 231-STOP) mutants were constructed by PCR with Vent DNA polymerase (New England Biolabs) by using the previously described mutant plasmids as templates for the PCR. The FLAG amino acids were inserted at the amino terminus of the protein by using the 5' primer LMPFLAG5 (5'-TAAATGGACTACAAAGACGATGACGACAAGGAACACGACCTTGAGAGG-3') and the 3' primer LMPFLAG3 (5'-ATCACGAGGAATTCAATGTGGCTTTTCAGCCTAGA-3'). PCR products were cloned into the *EcoRV* site of pcDNA3. All mutations were confirmed by sequence analysis with the Sequenase kit (U.S. Biochemicals).

**Transfections and CAT assays.** Transfections of C33A epithelial cells were carried out with  $5 \times 10^5$  cells per 60-mm-diameter dish with 5 to 7  $\mu$ g of total DNA and 10  $\mu$ l of Lipofectin according to the manufacturer's specifications (Gibco-BRL). One microgram of reporter plasmid ( $\kappa$ B-CAT) was transfected with 1  $\mu$ g of effector plasmid (e.g., LMP1, LMP1 mutants, and CD40). Full-length or deleted TRAF3-C (in the pSG5 vector) was transfected in fourfold excess to the effector plasmids. Cells were fed with serum-containing medium 24 h after transfection, and 24 h after feeding cells were washed in PBS, resuspended in 0.25 M Tris-HCl, and lysed by repeated freeze/thaw cycles. In some cases, transfected cells were stimulated with a 1:5,000 dilution of the anti-CD40 agonistic antibody (HB-14) 24 h after transfection. The HB-14 monoclonal antibody (Mab) was a kind gift of Thomas Tedder. Extracts were normalized for protein concentration and assayed for CAT activity as previously described (16, 57). Acetylated and nonacetylated forms of [ $^{14}$ C]chloramphenicol were separated by thin-layer chromatography and analyzed on a PhosphorImaging screen (Molecular Dynamics).

**Immunoblot analysis.** Protein extracts were made by solubilizing adherent cells in radioimmunoprecipitation assay buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], and 1% deoxycholate with protease inhibitors) with an equal volume of 2 $\times$  SDS sample buffer (adherent cells) or by resuspending suspension cells in PBS before adding equal volumes of 2 $\times$  SDS sample buffer (suspension cells). Extracts were boiled for 10 min, separated on SDS-7% polyacrylamide gels and transferred to supported nitrocellulose filters (Schleicher and Schuell) by using a Bio-Rad trans-blot wet electrophoretic 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 Mab (generous gift of David A. Thorley-Lawson) was used at a 1:10 dilution in BLOTTO for the detection of LMP1. The M5 anti-FLAG Mab (Sigma) was used at a 5  $\mu$ g/ml concentration for detection of LMP1 mutants. 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 (kind gift of H. Shelton Earp). Rabbit antiserum directed against TRAF3 (CRAF1-C20) and CD40 (C-20) were both used at concentrations of 1  $\mu$ g/ml according to the manufacturer's specifications (Santa Cruz Biotechnology, Inc.). Antiserum directed against the A20 protein was used at a 1:500 dilution (generous gift of Vishva Dixit). 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.

**RNA isolation and Northern blot analysis.** Total cellular RNA was isolated by directly lysing cells in guanidine isothiocyanate layered over CsCl and then centrifuging for 16 h in an ultracentrifuge. The guanidine isothiocyanate and CsCl were carefully removed, and the resulting pellet was resuspended in diethyl pyrocarbonate-treated H<sub>2</sub>O. For Northern blot analysis 25  $\mu$ g of total cellular RNA was electrophoresed through 1% agarose-formaldehyde gels and transferred to supported nitrocellulose (Schleicher and Schuell) overnight in 20 $\times$  SSPE (1 $\times$  SSPE is 0.18 M NaCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, and 1 mM EDTA [pH 7.7]). Northern blots were baked in a vacuum oven for 2 h, prehybridized for  $>2$  h (6 $\times$  SSPE, 10 $\times$  Denhardt's, 0.5% SDS, 50- $\mu$ g/ml denatured DNA, and 10- $\mu$ g/ml denatured RNA), and hybridized overnight (6 $\times$  SSPE, 1 $\times$  Denhardt's, 0.5% SDS, 50% formamide, and 50- $\mu$ g/ml denatured DNA) at  $>1 \times 10^6$  cpm/ml with random-primed probes labeled according to manufacturer's specifications (Promega, Madison, Wis.). The EGFR,  $\beta$ -actin, and A20 cDNAs used for probes were kindly provided by J. Schlessinger and S. Haskill.

#### RESULTS

**LMP1 mutants.** The LMP1 mutants used for this study were constructed to distinguish between the suggested signaling domains present in the protein (Fig. 1) (24, 39). The LMP1 protein consists of a 24-amino-acid domain located at the amino terminus, a membrane-spanning hydrophobic domain consisting of 6 transmembrane domains connected by short turns between amino acids 25 and 187, followed by a 200-amino-acid cytoplasmic domain at the carboxy terminus (12,

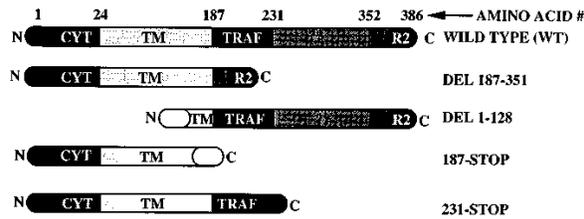


FIG. 1. Diagram of wild-type and mutant LMP1 proteins. The 386-amino-acid wild-type protein is divided into three major regions consisting of a 24-amino-acid cytoplasmic amino terminus (CYT), a membrane-spanning region consisting of six hydrophobic domains (TM) followed by a 200-amino-acid cytoplasmic carboxy-terminal region. The carboxy-terminal region can be further divided into the TRAF interaction domain between amino acids 187 and 231 and the distal activation domain between amino acids 352 and 386 (R2) which, like the TRAF interaction domain, is partially responsible for NF- $\kappa$ B activation. The DEL 187-351 mutant contains amino acid 352 fused in frame to amino acid 186. The DEL 187-351 mutant would retain the second activator domain but be unable to interact with the TRAFs. The DEL 1-128 construct is deleted for DNA encoding amino acids 1 to 122 such that translation begins at the methionine at amino acid 129. DEL 1-128 is deleted for the amino terminus and first four transmembrane domains. The 187-STOP and 231-STOP mutants contain stop codons following amino acids 187 and 231, respectively. The 187-STOP mutant is deleted for the entire carboxy-terminal domain, while the 231-STOP mutant contains the TRAF interaction domain.

19). The carboxy-terminal cytoplasmic domain is further divided into three regions. The proximal domain (amino acids 187 to 231) interacts with the TRAF molecules and mediates low-level NF- $\kappa$ B activation (9, 41). The distal domain (amino acids 352 to 386) encodes the major NF- $\kappa$ B activation domain mediated through interaction with unidentified signaling partners (24, 39, 41). The amino acids separating these two domains (amino acids 232 to 351) are not associated with a known function. From the LMP1 cDNA, the 595-nucleotide *Nco*I fragment was deleted to encode a protein fused at amino acids 186 and 352. This protein (DEL 187-351) is unable to interact with TRAF3 but retains the distal NF- $\kappa$ B activation domain. Deletion of the first 128 amino acids of LMP1 corresponds to a naturally occurring form that is expressed in some partially permissive cell lines (63). This protein (DEL 1-128) is inserted into all cell membranes but does not aggregate in the plasma membrane and is nontransforming (32, 63). The final two mutants used in the study were generated by inserting stop codons after amino acids 187 (187-STOP) and 231 (231-STOP). The 187-STOP mutant is deleted for the entire carboxy-terminal cytoplasmic domain, while the 231-STOP mutant retains the TRAF interaction domain.

**Activation of NF- $\kappa$ B by LMP1 and mutants in C33A epithelial cells.** The panel of mutants was assayed for NF- $\kappa$ B activation by using a reporter construct ( $\kappa$ B-CAT) that contains the NF- $\kappa$ B sites from the HIV-LTR (57). Wild-type LMP1 strongly activated the construct by greater than 10-fold over that of the vector control (Fig. 2). This effect was shown to be specific for NF- $\kappa$ B, since the same construct lacking the  $\kappa$ B sites (TATA-CAT) was unresponsive to LMP1. The mutant which had the first four transmembrane domains deleted (DEL 1-128) had very little transactivation activity, consistent with previous results (24). The mutant from which the entire carboxy terminus was deleted (187-STOP) also completely lacked the ability to activate NF- $\kappa$ B. However, the DEL 187-351 mutant had nearly wild-type activity (8.4-fold or 75% of wild type). The mutant that retains the TRAF interaction domain (231-STOP) had reduced but clearly evident activity (4.1-fold or <40% of wild type). These experiments indicate that two domains within the LMP1 protein are important for NF- $\kappa$ B ac-

tivation in C33A epithelial cells. The strongest domain is located between amino acids 352 and 386, and a second weaker domain overlaps the TRAF interaction domain between amino acids 187 and 231.

**Identification of LMP1 signaling domains required for EGFR and A20 induction.** In order to identify the regions of LMP1 essential for induction of the EGFR, stable cell lines expressing wild-type LMP1 and each of the LMP1 mutants were prepared and assayed by Western blot for EGFR expression (Fig. 3A). The ME-180 cervical carcinoma cell line which expresses high levels of the EGFR was included as a positive control for EGFR expression. As expected, wild-type LMP1 upregulated expression of the EGFR. Interestingly, the only mutant capable of upregulating expression of the EGFR was 231-STOP, which retained the TRAF interaction domain, while the mutant which retained the major NF- $\kappa$ B activation domain, DEL 187-351, did not induce expression of the EGFR. This revealed that NF- $\kappa$ B activation by LMP1 is not sufficient for induction of the EGFR. The DEL 1-128 and 187-STOP mutants also did not induce EGFR expression. Expression of LMP1 was detected in the EBV-infected CB5 cell line and in the C33A cell line expressing full-length wild-type LMP1. The mutant deleted for amino acids 1 to 128 was also detected with the S12 MAb (Fig. 3C). This mutant has been

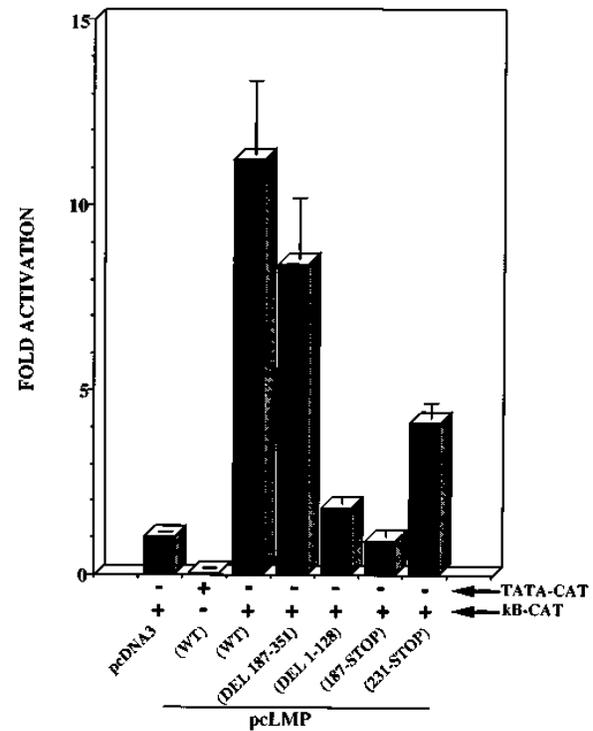


FIG. 2. Mutational analysis of LMP1 with respect to NF- $\kappa$ B activation. Wild-type LMP1 or each of the various LMP1 mutants (1  $\mu$ g) described in the legend for Fig. 1 were transfected into C33A cells along with a  $\kappa$ B-CAT reporter construct (1  $\mu$ g).  $\kappa$ B-CAT contains the  $\kappa$ B sites from the HIV-LTR linked to a minimal TATA box containing promoter. Wild-type LMP1 was also transfected with an identical reporter gene which was deleted for the  $\kappa$ B sites (TATA-CAT) to insure specificity for NF- $\kappa$ B activation. Cells were harvested 48 h after transfection, and CAT activity was quantitated as described in Materials and Methods. The results are shown as fold activation over vector (pcDNA3)-transfected control. The data represent three experiments performed in duplicate.

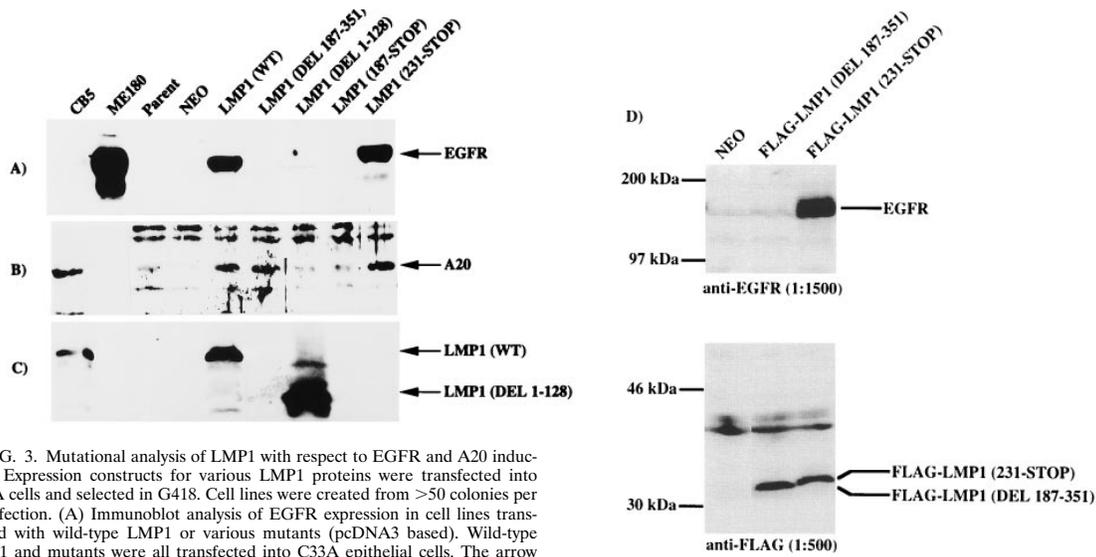


FIG. 3. Mutational analysis of LMP1 with respect to EGFR and A20 induction. Expression constructs for various LMP1 proteins were transfected into C33A cells and selected in G418. Cell lines were created from >50 colonies per transfection. (A) Immunoblot analysis of EGFR expression in cell lines transfected with wild-type LMP1 or various mutants (pcDNA3 based). Wild-type LMP1 and mutants were all transfected into C33A epithelial cells. The arrow depicts the 170-kDa EGFR detected by the ERCT antiserum. CB5, an EBV-infected lymphocyte cell line, and ME-180, an epithelial cell line, were used as controls. (B) Immunoblot analysis of A20 expression. Parallel blots containing the same panel of cell lines were probed with an A20 MAb. The arrow depicts the 85-kDa A20 protein. (C) Immunoblot analysis of LMP1 expression. Parallel blots containing the same cell lines were probed with the anti-LMP1 MAb S12. The 63-kDa wild-type LMP1 protein and the amino-terminal-truncated DEL 1-128 LMP1 mutant are indicated. Other LMP1 mutants are not recognized by the S12 antibody due to deletion of the S12 epitope. Smaller bands in the LMP1 wild-type-transfected cell lines probably represent translation initiation at internal methionines. (D) Immunoblot analysis of EGFR and FLAG-LMP1 mutant proteins are indicated. NEO, vector control transfected cells.

previously shown to lack the effects of wild-type LMP1, likely due to its inability to properly insert in the plasma membrane (32). Expression of the other mutants was not detected by Western blot due to deletion of the epitope for the S12 MAb in the other mutants (34). Expression of the LMP1 mutants in each cell line was confirmed by Northern blot analysis, and mutant LMP1 mRNAs were detected at equal or higher levels than the wild-type mRNA (data not shown).

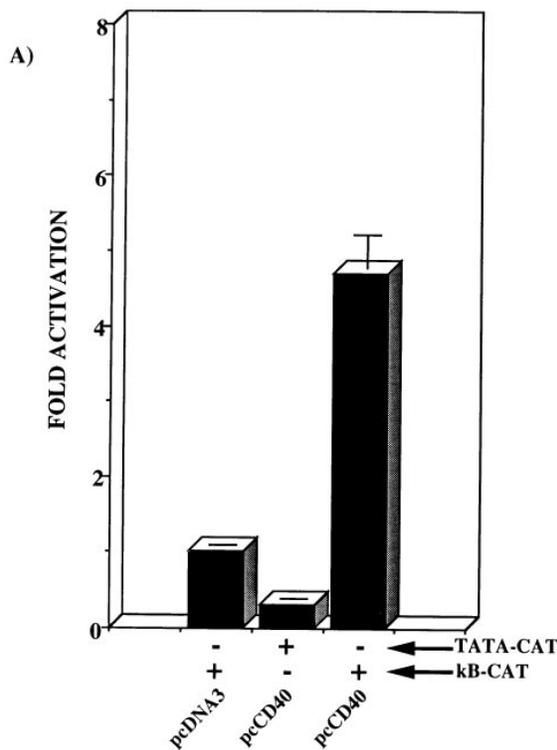
The cell lines expressing wild-type LMP1 and mutant LMP1 proteins were then analyzed by Western blotting for expression of the A20 protein. The EBV-infected CB5 cell line was used as a positive control for A20 expression (Fig. 3B). In contrast to EGFR induction, which was specific for the TRAF interaction domain of LMP1, induction of the NF- $\kappa$ B-regulated A20 gene was mediated by either of the two NF- $\kappa$ B activation domains of LMP1. Both the mutant which retains the TRAF interaction domain (231-STOP) and the mutant which retains the distal NF- $\kappa$ B activation domain (DEL 187-351) induced expression of A20. The two other mutants tested, 187-STOP and DEL 1-128, did not induce expression of A20, most likely reflecting the lack of NF- $\kappa$ B activation by these mutants. The 231-STOP mutant which had reduced NF- $\kappa$ B activation (<40% of wild type) in transient reporter assays (Fig. 2) actually induced higher levels of the A20 protein (Fig. 3B). These results suggest that both domains of LMP1 activate NF- $\kappa$ B sufficiently to induce expression of NF- $\kappa$ B responsive genes such as A20.

In order to ensure that mutations within the carboxy termi-

nus of LMP1 did not affect the expression or stability of mutant proteins, the two critical LMP1 mutants, DEL 187-351 and 231-STOP, were expressed with a FLAG epitope tag at the amino terminus of the proteins, and the expression of these mutants was analyzed by Western blot. FLAG-DEL 187-351 and FLAG-231-STOP were expressed at equal levels in the transfected C33A cells, indicating that deletions within this region of the LMP1 protein do not affect the expression or stability of the mutant proteins (Fig. 3D). Expression of the EGFR was then analyzed in these cell lines by Western blot. In confirmation of the previous results, only the FLAG-231-STOP mutant of LMP1 induced expression of the EGFR (Fig. 3D).

These results indicate that two domains within LMP1 are essential for engaging three distinct signaling pathways. First, the interaction of LMP1 with signaling molecules via the presence of the TRAF interaction domain induces expression of the EGFR (Fig. 3A and D). Second, the TRAF interaction domain also induces expression of the A20 gene due to the activation of NF- $\kappa$ B (Fig. 3B). Third, the interaction of LMP1 with signaling molecules via the presence of the distal domain (amino acids 352 to 386) activates NF- $\kappa$ B and induces A20 expression but is unable to induce EGFR expression (Fig. 3A, B, and D). These are the first data that identify an effect of the TRAF interaction domain that is distinct from NF- $\kappa$ B activation alone and suggest that TRAF signaling from LMP1 extends to activators of transcription other than NF- $\kappa$ B.

**Expression of CD40 in C33A epithelial cells activates NF- $\kappa$ B and induces expression of the EGFR.** The functional similarity between LMP1 and CD40, as well as the evidence that LMP1 and CD40 interact with the same set of signaling molecules, suggested that CD40 may also induce expression of the EGFR. The CD40 cDNA was subcloned into the pcDNA3 expression vector and tested for its ability to induce NF- $\kappa$ B, a function of CD40 that has previously been described (Fig. 4A). Expression of CD40 in C33A cells induced CAT activity 4.7-fold over the vector control. This induction was specific for the  $\kappa$ B sites as the control vector lacking the  $\kappa$ B recognition site was unaffected by CD40 expression (Fig. 4A). Stimulation of transfected cells with the anti-CD40 agonistic MAb HB-14 had very



little additional effect on further activation of the κB-CAT reporter plasmid (data not shown). This result is consistent with other studies where expression of either the type 2 TNFR (TNF-R2) or CD40 activated NF-κB in the absence of ligand (48). These results indicated that although CD40 is not expressed in C33A cells, members of the CD40 signaling complex (i.e., TRAF molecules) are present and capable of NF-κB activation when CD40 is overexpressed.

To determine the effects of CD40 overexpression on EGFR expression, stable C33A cell lines expressing CD40 were prepared. The EGFR was not expressed in either the parental or control vector-transfected cells, but was readily detected in the CD40-transfected cell line (Fig. 4B). Analysis of CD40 expression in the same cells confirmed the strong expression of CD40 in the transfected cells with little or no endogenous CD40 expression in the parental and vector control cell lines (Fig. 4C). These data indicated that CD40, like LMP1, induces expression of the EGFR.

**Expression of EGFR and A20 mRNAs in cell lines expressing LMP1 mutants and CD40.** To determine if LMP1 and CD40 induced the EGFR and A20 at the transcriptional level and to confirm the previous Western blot analyses, a Northern blot was prepared with 25 μg of total cellular RNA from cell lines expressing wild-type LMP1, LMP1 mutants, and CD40. The filter was cut in half, and the top half was hybridized with an EGFR cDNA probe and the lower half was hybridized with a cDNA representing the full-length A20 gene (Fig. 5A and B). Specific hybridization of the 10.5-kb EGFR mRNA was detected with the EGFR probe in cell lines expressing full-length LMP1, the mutant which retains the TRAF interaction domain (231-STOP) and CD40. These results are in complete agree-

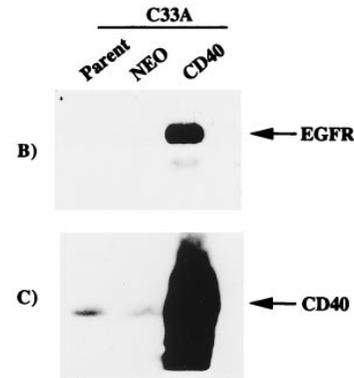


FIG. 4. CD40 activates NF-κB and induces expression of the EGFR. (A) Transactivation of NF-κB by CD40. CD40 (1 μg) was transfected with the κB-CAT reporter plasmid (1 μg). The control CAT reporter plasmid (TATA-CAT) was transfected in order to insure specificity of NF-κB activation. Cells were harvested 48 h after transfection, and CAT activity was quantitated as described in Materials and Methods. The results are shown as fold activation over vector (pcDNA3)-transfected control. The data represent three experiments performed in duplicate. (B) Immunoblot analysis of EGFR expression in CD40-expressing and control C33A epithelial cell lines. Cell lines were derived with either the control vector (pcDNA3) or CD40 expression vector (pcCD40) by expansion of >50 G418-resistant colonies. The 170-kDa EGFR detected by the ERCT antiserum is indicated by the arrow. (C) Immunoblot analysis of CD40 expression. Parallel samples to those analyzed in panel B were analyzed for CD40 expression. The 45-kDa CD40 molecule is indicated by the arrow.

ment with Western blot analyses shown in Fig. 3A and 4B. Hybridization was not detected in the cell lines expressing the DEL 187-351 mutant, which retains NF-κB activation, or the other mutants, indicating that the TRAF interaction domains of LMP1 and CD40 are responsible for the induction of EGFR expression at the mRNA level. In contrast, high levels of the 4.4-kb A20 mRNA were detected in the cell lines containing full-length LMP1, 231-STOP, DEL 187-351, and CD40. Interestingly, in confirmation of the Western blot analysis, the 231-STOP mutant again induced higher levels than the DEL 187-351 mutant, despite the fact that 231-STOP has reduced NF-κB activation in reporter gene assays. This result indicates that the 231-STOP mutant can activate NF-κB to sufficient levels to induce expression of the A20 gene in the context of the cellular genome. The Northern blot was reprobed with a β-actin cDNA to ensure equal loading and integrity of the RNA (Fig. 5C). These results indicate that LMP1 utilizes two mechanisms to induce gene expression: (i) induction of mRNA expression, such as EGFR, that involves a factor independent from NF-κB and (ii) activation of transcription dependent on NF-κB, such as A20, which can be mediated by both the TRAF interaction domain and the distal effector domain (amino acids 352 to 386).

**Negative regulation of signals from the TRAF interaction domain by TRAF3 overexpression.** Overexpression of full-length TRAF3 can block CD40- and TNF-R2-mediated activation of NF-κB in the 293 embryonic kidney cell line (48). Overexpression of an amino-truncated version of TRAF3 (TRAF3-C) blocked upregulation of CD23 following ligand-dependent activation of CD40 in the Ramos B-lymphoma cell line (6). These results suggest that TRAF3 may act as a negative regulator of the TRAF signaling pathway. Since LMP1 interacts with TRAF3, expression of endogenous TRAF3 and the effect of TRAF3 overexpression on LMP1-mediated activation of NF-κB was analyzed in the C33A epithelial cell line.

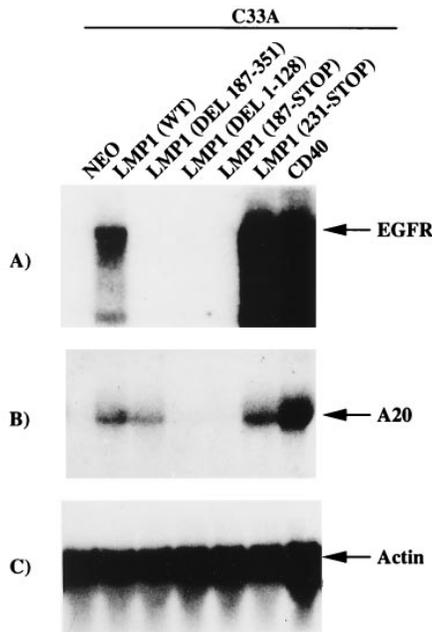


FIG. 5. Expression of EGFR and A20 specific mRNAs in C33A cell lines expressing LMP1 and mutants. (A) Total cellular RNA (25  $\mu$ g) was isolated from LMP1- and LMP1 mutant-expressing cell lines and analyzed by Northern blotting for EGFR expression with the EGFR cDNA probe. The 10.5-kb EGFR mRNA is indicated. (B) The lower half of the same blot shown in panel A was analyzed by Northern blotting for A20 expression with the A20 cDNA probe. The location of the 4.4-kb A20 mRNA is indicated. (C) The same blot was reprobed with a  $\beta$ -actin cDNA probe to demonstrate equal loading and integrity of the RNAs. NEO, vector control transfected cells.

TRAF3 expression was analyzed by reverse transcription-based PCR in C33A epithelial cells. TRAF3 was expressed in the parental cells, and expression of either LMP1 or CD40 did not change the level of endogenous TRAF3 expression (data not shown). These results indicate that the activation of NF- $\kappa$ B by LMP1 and CD40 does not involve changes in the level of TRAF3 expression and suggest that modulation of TRAF3 function by LMP1 and CD40 may be involved in NF- $\kappa$ B activation.

To further define the role of TRAF signaling on LMP1 function, the effect of TRAF3 overexpression on the activation of NF- $\kappa$ B was analyzed in transient assays. The use of NF- $\kappa$ B-dependent reporter gene assays has proven useful in understanding TRAF signaling, due to the ability to easily manipulate expression levels of the various proteins. The  $\kappa$ B-CAT construct was cotransfected into C33A cells with expression vectors for wild-type LMP1, LMP1 mutants, or CD40, as well as a fourfold excess of expression vectors for the full-length TRAF3 (amino acids 1 to 568) or amino-truncated TRAF3-C (amino acids 347 to 568) (Fig. 6). Since CD40 activation of NF- $\kappa$ B in C33A cells was augmented only minimally by the addition of anti-CD40 MAb, all experiments with CD40 were done in the absence of antibody (Fig. 4A and data not shown). TRAF3-C lacks the amino-terminal ring finger and zinc finger domains and most of the coiled-coil domain but retains the ability to interact with LMP1 and CD40 (6, 41). Overexpression of TRAF3 and TRAF3-C in the absence of LMP1 or CD40 was analyzed in order to determine what effect, if any, these molecules had in cells containing inactive NF- $\kappa$ B. In the

absence of LMP1, expression of the full-length TRAF3 did not activate NF- $\kappa$ B, although expression of TRAF3-C alone did activate NF- $\kappa$ B slightly (about 3.5-fold). Expression of full-length TRAF3 did not affect NF- $\kappa$ B activation by wild-type LMP1, while expression of TRAF3-C reduced NF- $\kappa$ B activation by 50%. The LMP1 mutant that lacks the TRAF interaction domain but retains the distal NF- $\kappa$ B activation domain (DEL 187-351) was unaffected by expression of either full-length TRAF3 or TRAF3-C, indicating that this mutant activates NF- $\kappa$ B independently of TRAF3. However, the mutant retaining the TRAF interaction domain (231-STOP) was severely blocked for NF- $\kappa$ B activation by TRAF3. Full-length TRAF3 reduced NF- $\kappa$ B activation by the 231-STOP mutant by 75%, while expression of the TRAF3-C inhibited 67% of NF- $\kappa$ B activation. Western blot analysis demonstrated that increasing amounts of TRAF3 or TRAF3-C did not affect LMP1 expression, indicating that TRAF3 suppression of NF- $\kappa$ B activation is not due to decreased LMP1 expression (data not shown). This indicated that the induction of NF- $\kappa$ B mediated by the TRAF interaction domain of LMP1 is negatively regulated by overexpression of TRAF3 in C33A epithelial cells. Activation of NF- $\kappa$ B by CD40 expression was also reduced by full-length TRAF3 and TRAF3-C expression in the C33A cells.

To determine if TRAF3 also blocked induction of the EGFR by LMP1 and CD40, several attempts were made to establish cell lines expressing LMP1 and either TRAF3 or TRAF3-C. Cell lines that expressed full-length TRAF3 could not be established, suggesting that stable expression of TRAF3 is toxic to epithelial cells (data not shown). C33A cell lines expressing LMP1 under the control of the CMV promoter or the K14 promoter in combination with TRAF3-C could be

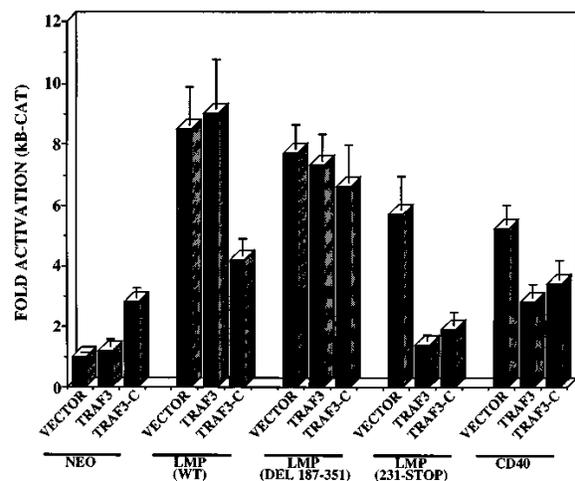


FIG. 6. TRAF3 overexpression inhibits NF- $\kappa$ B activation by the TRAF interaction domain of LMP1. Wild-type LMP1, NF- $\kappa$ B-activating LMP1 mutants DEL 187-351 and 231-STOP, and CD40 (1  $\mu$ g) were transfected into C33A cells along with a  $\kappa$ B-CAT reporter construct (1  $\mu$ g). To determine the effect of TRAF3 on LMP1 transactivation of  $\kappa$ B-CAT, 4  $\mu$ g of vector control, full-length TRAF3, or amino-truncated TRAF3 (TRAF3-C) was also transfected along with the other plasmids. "VECTOR" represents the parental vector (pSG5) used for expression of the TRAF3 proteins. "NEO" represents the parental vector (pCDNA3) used for expression of the various LMP1 proteins and CD40. Cells were harvested 48 h after transfection, and CAT activity was quantitated as described in Materials and Methods. The results are shown as fold activation over controls transfected with vector (pCDNA3 and pSG5) alone. The data represent five independent experiments performed in duplicate.

established. Western blot analysis indicated that TRAF3-C was expressed at low levels in these cell lines. LMP1 also was expressed in the same cell lines, although the K14-LMP1 cell line expressed higher levels of LMP1 than the CMV-LMP1 cell line. Interestingly, expression of the EGFR was blocked in the CMV-LMP1 cell line but not in the K14-LMP1 cell line (data not shown). These results suggest that the relative levels of LMP1 and TRAF3 molecules contribute to activation or repression of this pathway and that LMP1 signaling through the TRAF interaction domain is negatively regulated by TRAF3 expression.

## DISCUSSION

The EBV LMP1 protein is one of several latently expressed genes essential for growth transformation of B lymphocytes *in vitro* (25, 28, 29). The viral protein is also important in the development of EBV-infected epithelial malignancies, as it is detected in all cells of early preinvasive NPC lesions (46). LMP1 affects cellular growth control and differentiation of epithelial cells *in vitro* (8, 11, 23, 68). The profound ability of LMP1 to alter cellular gene expression is likely to mediate these effects (10, 17, 18, 24, 31, 42, 64). However, the underlying molecular events that enable LMP1 to induce these changes in epithelial cells are largely unknown.

A key property of LMP1 is the ability to interact with members of the TRAF signaling pathway (9, 41). The TRAF molecules interact with the TNFR superfamily of receptors and mediate downstream events in response to stimulation with ligand (3, 58). This diverse group of membrane-bound receptors lack intrinsic kinase activity and activate signal transduction pathways through interaction with the TRAF molecules (48, 58). Individual members of the TNFR superfamily interact with different combinations of TRAF molecules and adapter proteins, such as TRADD, to either activate NF- $\kappa$ B or induce cellular death (21). The TRAFs share a highly conserved C-terminal domain which mediates interactions between various TRAF family members and membrane-bound receptors. TRAF1 and TRAF2 were identified in coimmunoprecipitations with the 75-kDa TNF-R2 (49). TRAF3 was identified in a yeast two-hybrid analysis by its ability to interact with the cytoplasmic domains of both LMP1 and the CD40 receptor (6, 22, 41, 52). EBV induces expression of the TRAF1 protein, and both TRAF1 and TRAF2 also interact with the LMP1 TRAF interaction domain (9, 41). LMP1 constitutively aggregates in the cell membrane and may activate TRAF signaling pathways by allowing TRAF molecules to cluster at the cell membrane, which is similar to ligand activation of CD40 or the TNF-R2. TRAF2 is likely to be a positive mediator of TRAF signaling as overexpression of TRAF2 was subsequently shown to activate NF- $\kappa$ B independently of receptor aggregation (48). This activation was dependent on the amino-terminal ring finger domain, since expression of an amino-truncated TRAF2-C (amino acids 87 to 501) was nonfunctional.

Evidence suggests that in some cells TRAF3 may be a negative regulator of signaling through both the TNF-R2 and CD40. Overexpression of either the TNF-R2 or CD40 in the human embryonic kidney 293 cell line activates NF- $\kappa$ B, and this activation can be blocked by expression of TRAF3, while an amino-terminal-deleted version of TRAF3 (TRAF3-C) could block the induction of CD23 following ligation of CD40 (6, 48). Overexpression of TRAF3 in BJAB cells expressing LMP1 displaces TRAF1 and TRAF2 from the membrane-associated signaling complex, suggesting that negative regulation by TRAF3 involves disruption of a LMP1/TRAF1/TRAF2 complex important for NF- $\kappa$ B activation (9). LMP1 may mod-

ulate TRAF signal transduction events by binding negative regulators of the TRAF pathway like TRAF3.

Three lines of evidence presented in this study indicate that LMP1 mediates the induction of EGFR expression through interaction with the TRAF signaling pathway. First, although both the mutant which retains the TRAF interaction domain (231-STOP) and the LMP1 mutant (DEL 187-351) activate NF- $\kappa$ B, only the LMP1 mutant (231-STOP) induces expression of the EGFR. This also indicates that TRAF-mediated induction of the EGFR involves an additional pathway distinct from NF- $\kappa$ B activation. Second, CD40 which signals through TRAF molecules can also induce expression of the EGFR. Third, signaling from the TRAF interaction domain is inhibited by TRAF3-C overexpression. The relative levels of LMP1 and TRAF3-C expression are apparently critical for the induction of the EGFR. Thus, the cell line that expressed high levels of LMP1 had induced expression of the EGFR in the presence of TRAF3-C, while the cell line that expressed a low level of LMP1 was inhibited for EGFR induction by TRAF3-C expression. Together, these results indicate that there is a TRAF signal transduction pathway distinct from NF- $\kappa$ B that is activated by LMP1 or CD40 expression and it can induce EGFR expression.

Interestingly, activation of the 55-kDa type 1 TNFR (TNF-R1) with TNF- $\alpha$  can induce expression of the EGFR in pancreatic cell lines (26, 27, 53). Recent reports indicated that all of the known TRAF molecules can interact with the TNF-R1 via the adapter molecule TRADD (21). The data presented in this study suggests that TRAF signaling resulting from activation of the TNF-R1, TNF-R2, or CD40 or from expression of LMP1 results in the induction of the EGFR.

The results presented here support a model shown in Fig. 7. In the absence of either LMP1 or activated CD40, TRAF signaling molecules are present but are unable to activate signaling pathways resulting in A20 or EGFR expression (Fig. 7A). Expression of LMP1 in C33A epithelial cells then engages three distinct signaling pathways (Fig. 7B). The TRAF interaction domain in LMP1 activates the transcription factor NF- $\kappa$ B resulting in induction of genes including the NF- $\kappa$ B-regulated A20 gene. The TRAF interaction domain also induces expression of the EGFR through the activation of a pathway distinct from NF- $\kappa$ B activation alone. The distal LMP1 domain initiates the third signaling event. The distal domain, which does not interact with the TRAF molecules, activates NF- $\kappa$ B, resulting in the induction of A20 expression, but has no effect on EGFR expression. The presence of two domains in LMP1 that can activate NF- $\kappa$ B through TRAF-dependent and through TRAF-independent pathways suggests that NF- $\kappa$ B activation is carefully regulated during viral transformation. It will be of interest to determine if both NF- $\kappa$ B activation domains induce the same genes, as is the case for A20, or whether the two domains induce different NF- $\kappa$ B complexes resulting in the induction of distinct genes. It is presently unknown if TRAF3 is present in the NF- $\kappa$ B-activating LMP1-TRAF complexes, but it is clear that the endogenous level of TRAF3 does not block the ability of LMP1 to activate NF- $\kappa$ B and induce EGFR expression. When TRAF3 is overexpressed in this system, activation of NF- $\kappa$ B and presumably EGFR induction are blocked (Fig. 7C). Overexpression of TRAF3 can compete with molecules like TRAF1 and TRAF2 for binding to LMP1, effectively blocking the TRAF signal transduction pathway activated by LMP1 (9). TRAF3 may have a modulatory role so that excessive activation of the TRAF pathway does not occur. The activation of NF- $\kappa$ B by the distal domain is unaffected by TRAF3 overex-

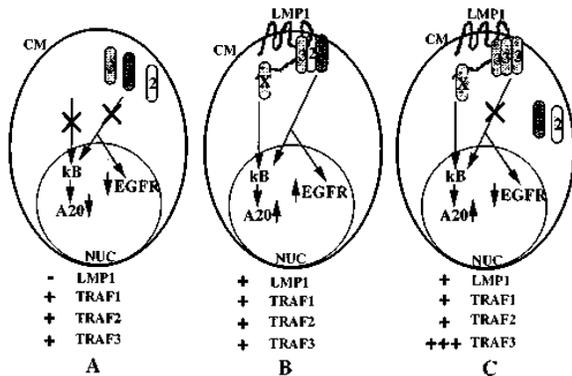


FIG. 7. A model for the induction of EGFR expression and activation of NF- $\kappa$ B by LMP1. +, low endogenous level of TRAF expression; +++, high level of TRAF3 overexpression. In the absence of LMP1, the TRAF signaling molecules are present but unable to cluster and activate downstream signaling pathways. This results in the lack of EGFR expression and lack of NF- $\kappa$ B activation and A20 expression (A). In the presence of LMP1 three signaling pathways are activated (B). Two involve the TRAF interaction domain and the third involves undefined signaling partners. LMP1 clustering in the cell membrane allows TRAF molecules such as TRAF2 to cluster and activate downstream signaling pathways. The activation of these pathways results in induction of EGFR expression as well as NF- $\kappa$ B activation and induction of A20 expression. Overexpression of TRAF3 negatively affects LMP1 function, resulting in a block of signaling events from the TRAF interaction domain (C). TRAF3 may interfere with the ability of LMP1 to interact with molecules like TRAF1 and TRAF2 at the cell membrane, thereby blocking TRAF signaling. CM, cell membrane; NUC, nucleus; X, undefined signaling partners;  $\kappa$ B, NF- $\kappa$ B.

pression, reflecting the fact that this activation results from interaction with undefined cellular signaling partners.

These data further define the method of transcriptional activation induced by the EBV LMP1 oncoprotein. The data indicate that LMP1 induces different sets of genes through either activation of the TRAF signaling pathway, mediated by amino acids 187 to 231, or through TRAF-independent activation of NF- $\kappa$ B, mediated by amino acids 352 to 386. These results also indicate that activation of TNFR family molecules, such as CD40, regulate expression of the EGFR through activation of the TRAF pathway. The EGFR and other genes regulated by activation of the TRAF pathway are likely to mediate the oncogenic effects of LMP1 and CD40 expression in epithelial cells.

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