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Epstein-Barr Virus Latent Membrane Protein 1 Modulates Distinctive NF-κB Pathways through C-Terminus-Activating Region 1 To Regulate Epidermal Growth Factor Receptor Expression▽

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Epstein-Barr Virus (EBV) latent membrane protein 1 (LMP1) is required for EBV B-lymphocyte transformation, transforms rodent fibroblasts, and can induce lymphoma and epithelial hyperplasia in transgenic mice. Two domains have been identified within the intracellular carboxy terminus that can activate NF-κB, C-terminus-activating region 1 (CTAR1) and CTAR2, through interactions with tumor necrosis receptor-associated factors (TRAFs). CTAR1 can activate both the canonical and noncanonical NF-κB pathways and has unique effects on cellular gene expression. The epidermal growth factor receptor (EGFR) is highly induced by LMP1-CTAR1 in epithelial cells through activation of a novel NF-κB form containing p50 homodimers and Bcl-3. To further understand the regulation of NF-κB in CTAR1-induced EGFR expression, we evaluated the ability of CTAR1 to induce EGFR in mouse embryonic fibroblasts (MEFs) defective for different NF-κB effectors. CTAR1-mediated EGFR induction required the NF-κB-inducing kinase (NIK) but not the IκB kinase (IKK) complex components that regulate canonical or noncanonical NF-κB pathways. CTAR1-mediated induction of nuclear p50 occurred in IKKβ-, IKKγ-, and NIK-defective MEFs, indicating that this induction is not dependent on the canonical or noncanonical NF-κB pathways. EGFR and nuclear p50 were expressed at high levels in TRAF2$^{-/-}$ fibroblasts and were not induced by CTAR1. In TRAF3$^{-/-}$ MEFs, CTAR1 induced nuclear p50 but did not affect basal levels of STAT3 serine phosphorylation or induce EGFR expression. EGFR was induced by LMP1 in TRAF6$^{-/-}$ MEFs. These findings suggest that this novel NF-κB pathway is differentially regulated by TRAF2 and TRAF3, and that distinct interactions of LMP1 and its effectors regulate LMP1-mediated gene expression.

Epstein-Barr virus (EBV) is a human gammaherpesvirus that infects more than 95% of the world population and is associated with multiple malignancies, including Hodgkin's disease (HD), NK or T-cell lymphoma, Burkitt's lymphoma (BL), posttransplant lymphoproliferative disease (PTLD), gastric carcinoma (GAC), and nasopharyngeal carcinoma (NPC) (43). Latent membrane protein 1 (LMP1) is considered the EBV oncogene and is essential for EBV-mediated B-cell transformation (20). Moreover, LMP1 can transform rodent fibroblast cells, and LMP1-transgenic mice develop B-cell lymphoma and epithelial hyperplasia (23, 32, 55, 56). LMP1 is expressed in multiple EBV-associated malignancies and acts as a constitutively active tumor necrosis factor receptor (TNFR) by recruiting TNFR-associated factors (TRAFs) to the cell membrane (21, 50). Numerous cellular genes have been shown to be induced by LMP1, including ICAM-1, TRAF1, A20, Id1, Id3, Bcl-2, Bcl-3, and epidermal growth factor receptor (EGFR) (24, 25, 36, 45, 49). LMP1 has two major signaling domains, C-terminus-activating region 1 (CTAR1) and CTAR2, which bind different TRAFs and activate distinct signaling pathways. CTAR1 recruits TRAF1, -2, -3, and -5 and uniquely activates noncanonical nuclear factor κB (NF-κB), phosphatidylinositol 3-kinase (PI3K)-Akt, and the mitogen-activated protein kinase (MAPK) pathways. CTAR2 recruits TRAF2 and TRAF6 through adaptors TRADD and BS69 to activate canonical NF-κB and c-Jun N-terminal kinase (JNK) signaling pathways (8, 18, 32, 33, 42). CTAR1 is required for LMP1-mediated fibroblast transformation and for B-lymphocyte transformation while CTAR2 is dispensable (32, 33).

NF-κB is a transcription factor family whose members dimerize and bind to κB sites within the promoter/enhancers to regulate transcription of genes that affect a variety of biological processes, including cell cycle progression, apoptosis, differentiation, inflammation, angiogenesis, and cell proliferation (15, 34). The NF-κB family consists of five members, p50, p52, p65 (RelA), RelB, and c-Rel, all of which share a Rel homology domain responsible for dimerization and DNA binding. The transcription activation domain required for gene regulation is found in p65, RelB, and c-Rel. The activation of NF-κB is tightly regulated through interactions with inhibitors of NF-κB (IκBs), which include p105 (precursor of p50), p100 (precursor of p52), IκBα, IκBβ, IκBγ, IκBε, IκBζ, and Bcl-3. IκBs function mainly by sequestering inactive NF-κB dimers in the cytoplasm to prevent their activity. Upon receiving an extracellular stimulus, such as binding of tumor necrosis factor alpha (TNF-α) to its receptor, activation of a kinase cascade that includes IκB kinase alpha (IKKα), IKKβ, and IKKγ (NEMO) results in the phosphorylation of IκBs and leads to their ubiquitination and degradation. NF-κB members are

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then released into the nucleus for transcriptional regulation. In the canonical NF-κB pathway, an IKK complex consisting of IKKα, IKKβ, and IKKγ is activated and results in IKKα/IKKβ-mediated phosphorylation and degradation of IκBα. Degradation of IκBα releases multiple NF-κB dimers, primarily p50/p65, into the nucleus. In the noncanonical NF-κB pathway, NF-κB-inducing kinase (NIK) phosphorylates and activates IKKα in an IKKβ/γ-independent manner. Activated IKKα phosphorylates p100 and triggers proteasome-mediated processing of p100 to p52, liberating p52-containing NF-κB dimers such as p52/RelB into the nucleus. Due to the very different mechanisms of activation, canonical and noncanonical NF-κB pathways likely regulate distinctive target genes. In addition to participating in both NF-κB pathways, IKKs and NIK have also been reported to phosphorylate many other proteins besides the IκBs. For example, IKKα and IKKβ can both directly phosphorylate p65 (41). IKKα has also been implicated in the phosphorylation of several nuclear substrates, including SMRT, histone H3, and CBP (16, 17, 58). NIK was recently suggested to function as a serine kinase of STAT3 (39). The aberrant regulation of NF-κB activation pathways has been implicated in development of many human cancers (34, 44).

Initial studies characterizing LMP1 activation of NF-κB showed that although CTAR2 induced greater NF-κB activation as determined using reporter assays, CTAR1 induced more complex NF-κB signaling detected by electrophoretic mobility shift assay (EMSA) (19, 35, 52). Subsequent studies revealed that CTAR2 only activates the canonical NF-κB pathway, whereas CTAR1 can activate both the canonical and noncanonical pathways (1, 9, 30, 46). LMP1-CTAR1 can transcriptionally activate EGFR expression, and this ability was shown to be mediated through a unique NF-κB complex containing p50 homodimers and Bcl-3 (24, 36, 53). The induction of this transcription complex requires activation of STAT3 by CTAR1 to increase Bcl-3 expression. Although it is considered a member of the IκB family, Bcl-3 contains a transactivating domain and can bind to p50 and p52 homodimers to induce their transcriptional activating potential (4, 13).

To determine if NF-κB-regulated EGFR expression reflected the activation of the canonical or noncanonical NF-κB pathway, the effects of LMP1-CTAR1 were analyzed in genetically engineered mouse embryonic fibroblasts (MEFs). LMP1-CTAR1-mediated upregulation of EGFR was independent of canonical or noncanonical regulation, as it did not require IKKα, IKKβ, or IKKγ, but was dependent on NIK. In addition, TRAF2 and TRAF3, but not TRAF6, were required for LMP1-CTAR1-mediated induction of EGFR in MEF cells. These data indicate that in addition to canonical and noncanonical NF-κB pathways, LMP1 also induces unique NF-κB complexes through CTAR1 to regulate target gene expressions.

MATERIALS AND METHODS

Reagents and cell culture. C33A cervical carcinoma cells were cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (Sigma) and antibiotic/antimycotic (Gibco) at 37°C with 5% CO_2. Wild-type (WT), IKKα$^{-/-}$, IKKβ$^{-/-}$, IKKγ$^{-/-}$, NIK$^{aly/aly}$, TRAF2$^{-/-}$, and TRAF6$^{-/-}$ mouse embryonic fibroblast (MEF) cells were kindly provided by Elliot Kieff (Harvard University, Boston) and were immortalized by being infected with a human papillomavirus 16 E6/E7 retrovirus to make MEF cells susceptible for transfection or transduction (7, 26, 29–31, 47). TRAF3$^{-/-}$ MEF cells were obtained from Michael Karin (University of California at San Diego) and were spontaneously immortalized by continuous passaging (14). Immortalized MEF cells were maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (Sigma) and antibiotic/antimycotic (Gibco) at 37°C with 5% CO_2. To inhibit proteasome activity, 1 to 10 μM MG132 (26S inhibitor) or *clasto*-lactacystin β-lactone (20S inhibitor) (Calbiochem) was added to ~90% confluent cells and left for 5 h before preparation of cell lysates.

Plasmids. Generation of plasmid constructs expressing both Myc-tagged (pCDNA3) and hemagglutinin (HA)-tagged (pBabe) full-length LMP1, LMP1-CTAR1 (which contains amino acids [aa] 1 to 231 of LMP1), and LMP1-CTAR2 (which has aa 187 to 351 of LMP1 deleted) was described previously (10). Myc-tagged and HA-tagged LMP1 constructs contain neomycin and puromycin resistance cassettes, respectively. HA-tagged LMP1, LMP1-CTAR1, and LMP1-CTAR2 were also subcloned into pCDNA3 vector with a zeocin resistance cassette by digesting pBabe-LMP1/CTAR1/CTAR2 with BamHI/EcoRI and ligated to BamHI/EcoRI-digested pCDNA3.1-zeocin vector (Invitrogen). Wild-type and dominant negative NIK constructs were kindly provided by Christian Jobin (University of North Carolina at Chapel Hill) and Elliot Kieff (Harvard University).

Retrovirus production and transduction. Recombinant retrovirus production and transduction were performed as previously described to transduce full-length LMP1, CTAR1(1–231), CTAR2(d187-351), or pBabe vector control (24). Briefly, ~60 to 80% confluent 293T cells in 100-mm plates were triply transfected using FuGEGE6 transfection reagent (Roche) according to the manufacturer's instruction with 5 μg pBabe (vector), pBabe-HA-LMP1, pBabe-HA-CTAR1, or pBabe-HA-CTAR2 and 5 μg pVSV-G- and 5 μg pGag/Pol-expressing plasmids. After 24 h of incubation at 37°C, the culture media were replaced with fresh media and the cells were transferred to 33°C for another 24 h of incubation. Cell supernatants were centrifuged at 1,000 × *g* for 5 min to remove cell debris, and virus-containing supernatant was collected and stored in −80°C if not immediately used. Cells to be transduced were grown to ~70 to 80% confluence and then transduced with virus-containing supernatant with 4 μg/ml Polybrene for 24 to 48 h at 37°C.

Generation of stable cell lines. C33A stable cell lines expressing CTAR1 (1–231) or vector control pBabe were established by retroviral transduction followed by selection and passages in the presence of 1 μg/ml puromycin (Sigma). IKKβ$^{-/-}$ and NIK$^{aly/aly}$ MEF cells stably expressing CTAR1 were generated by transducing cells with pBabe- and CTAR1-containing retrovirus solution with 4 μg/ml Polybrene for 24 to 48 h at 37°C, followed by selection and passages in the presence of 1 μg/ml puromycin. IKKα$^{-/-}$ and IKKγ$^{-/-}$ MEF cells stably expressing CTAR1 were made by transfecting cells with Myc-tagged pCDNA3 vector or pCDNA3-CTAR1 construct using FuGEGE6 transfection reagent for 48 h at 37°C, followed by selection and passages in the presence of 0.8 mg/ml G418 (Mediatech). Stable LMP1- and CTAR1-expressing TRAF2$^{-/-}$ and TRAF6$^{-/-}$ MEF cells were generated by transfecting cells with HA-tagged pCDNA3.1 vector, pCDNA3.1-LMP1, or pCDNA3.1-CTAR1 construct using FuGEGE6 transfection reagent for 48 h at 37°C, followed by selection and passages in the presence of 400 μg/ml zeocin (Invitrogen).

Fractionation of cells. Cells were fractionated as previously described (24). Briefly, after cultured cells reached ~90% confluence, cells were scrape harvested, washed once with cold phosphate-buffered saline (PBS) (Gibco), and centrifuged at 1,000 × *g* for 5 to 10 min to get cell pellets. Whole-cell lysates were made by lysing cells with radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% sodium dodecyl sulfate [SDS], 0.1% deoxycholic acid) supplemented with phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate (Na_3VO_4), and protease and phosphatase inhibitor cocktails (Sigma). After incubation at 4°C for 15 min, lysates were then clarified by centrifugation at 13,000 rpm and 4°C for 15 min, and supernatants containing whole-cell lysates were transferred to new tubes. Nuclear extracts were made by lysing initial cell pellets in a hypotonic buffer (20 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA) supplemented with PMSF, Na_3VO_4, and protease and phosphatase inhibitor cocktails (Sigma) for 15 min on ice. Nonidet P-40 was then added to a final concentration of 1%, followed by 1 min of vortexing. Nuclei were pelleted by low-speed centrifugation at 2,000 rpm for 10 min at 4°C, and the supernatant was collected as the cytoplasmic fraction. The nuclear fraction was purified using the Optiprep reagent (Sigma) as directed by the manufacturer. Nuclei were lysed with nuclear extraction buffer (20 mM Tris-HCl [pH 8.0], 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, PMSF, Na_3VO_4, and protease and phosphatase inhibitor cocktails [Sigma]) with the salt concentration adjusted to 400 mM with 5 M NaCl. All lysates were stored at −80°C.

Western blot analysis. The protein concentration in cell lysates was determined using Bio-Rad DC protein assay system according to the manufacturer's

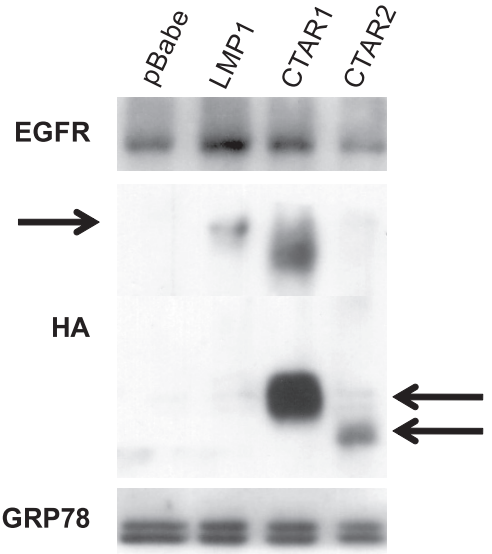

FIG. 1. EBV LMP1-CTAR1 mediates mild induction of EGFR expression in mouse embryonic fibroblast (MEF) cells. Control vector pBabe, full-length LMP1, truncated LMP1 containing only CTAR1, or truncated LMP1 containing only CTAR2 was transduced into wild-type MEF cells. Expression of EGFR and LMP1 derivatives was analyzed by immunoblotting with EGFR and HA tag antibodies, respectively. Expression of GRP78 was measured by immunoblotting as a loading control. Arrows indicate the sizes of different LMP1 deletion constructs. The results shown here are representative of three independent experiments.

instructions. Equal amounts of protein were used for SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to Optitran (Schleicher and Schuell) for Western blot analysis. Primary antibodies used included anti-p50 (Abcam), anti-p65 (RelA), anti-NIK, anti-RelB, anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase), anti-GRP78, anti-PARP [poly(ADP-ribose) polymerase] (Santa Cruz), anti-emerin, anti-phospho-STAT3 (Ser 727 and Tyr 705) (Cell Signaling), anti-β-catenin (BD Biosciences), and anti-HA tag (Covance). A rabbit antiserum raised against the carboxyl-terminal 100 amino acids of the EGFR fused to glutathione *S*-transferase was kindly provided by H. Shelton Earp (University of North Carolina at Chapel Hill) and used to detect total EGFR expression. S12 monoclonal antibody against LMP1 was a kind gift of David A. Thorley-Lawson (Tufts University). Secondary antibodies used to detect bound proteins include horseradish peroxidase-conjugated anti-mouse and anti-rabbit (Amersham Pharmacia) and anti-goat (Dako) antibodies. After treatment with secondary antibodies, blots were developed using the Pierce SuperSignal West Pico chemiluminescence system followed by exposure to film (ISCBioexpress).

RESULTS

LMP1 effects on EGFR expression in mouse embryonic fibroblast cells. To assess the induction of EGFR expression by LMP1 in mouse embryonic fibroblast (MEF) cells, full-length LMP1, LMP1-CTAR1, and LMP1-CTAR2 were expressed using the pBabe retroviral vector and transduced into wild-type MEF cells. The expression of LMP1, deletion mutants, and EGFR was determined by immunoblotting (Fig. 1). A higher-molecular-weight band was consistently detected in the CTAR1 deletion mutant, which may represent a nondenatured oligomer of LMP1. EGFR was readily detected in the WT MEFs, and both LMP1 and LMP1-CTAR1 increased EGFR expression, while LMP1-CTAR2 alone did not affect it, similar to previous reports (24, 53). The induction of EGFR by LMP1 was less in the MEF cells than what was previously shown in the C33A cells, a difference that likely reflects the higher basal levels of EGFR expression in the MEFs.

CTAR1-mediated EGFR upregulation is NIK dependent, but NIK is not sufficient to mimic LMP1-mediated EGFR induction. CTAR1 of LMP1 activates both the canonical and noncanonical NF-κB pathways to activate distinct NF-κB complexes, including p50/65, p52/p50, p52/RelB, and p50/50 (35, 42). The activation of canonical or noncanonical NF-κB is linked to complexes that contain different members of the inhibitor of NF-κB kinase kinases (IKK). The canonical pathway requires IKKα, IKKβ, and in some instances IKKγ, while the noncanonical pathway requires the activation of IKKα resulting from phosphorylation by the NF-κB-inducing kinase, NIK. The effects of expression of LMP1 or CTAR1 on EGFR and serine and tyrosine phosphorylation of STAT3 were evaluated in MEF cells lacking these regulators of NF-κB pathway (Fig. 2). LMP1 or LMP1-CTAR1 induced EGFR protein in IKKα-, IKKβ-, and IKKγ-defective MEF cells. As these IKKs are required for activation of the canonical NF-κB pathway, these data indicate that LMP1-mediated EGFR upregulation is not dependent on the canonical NF-κB pathway. Moreover, since IKKα is the critical regulator in the noncanonical NF-κB pathway, the result also reveals that the noncanonical NF-κB pathway is not required for LMP1-mediated EGFR induction. Interestingly, EGFR expression was not induced by CTAR1 in $NIK^{aly/aly}$ MEF cells. These results suggest that LMP1 induces EGFR through an IKKα-independent but NIK-specific pathway.

Activation of STAT3 by LMP1 is required for induction of Bcl-3 and EGFR expression (24). Expression of LMP1 increased serine phosphorylation of STAT3 in wild-type and IKKα- and IKKγ-defective MEF cells but not in IKKβ-defective or $NIK^{aly/aly}$ MEFs, both of which had high basal levels. Elevated tyrosine phosphorylation was detected in wild-type, IKKγ-defective and $NIK^{aly/aly}$ cells but not in IKKα- or IKKβ-defective MEF cells. Importantly, the effects of LMP1 on EGFR and STAT3 were not linked to the canonical or noncanonical NF-κB pathways but were linked to the presence of NIK.

A previous study showed that NIK could function as a serine kinase of STAT3; however, the high basal levels of serine-phosphorylated STAT3 in NIK-defective cells indicate that other kinases may also phosphorylate STAT3 (39). To confirm the requirement for NIK in LMP1-induced EGFR expression, CTAR1-expressing C33A cells were transiently transfected with a dominant negative NIK construct (DNNIK), DNNIK with an aly mutation (G860R; NIK-aly), or kinase-dead NIK [NIK(K-A)] for 48 h, and the expression levels of EGFR and serine-phosphorylated STAT3 were analyzed by immunoblotting (Fig. 3A). DNNIK and NIK-aly express the C terminus of NIK, which contains the TRAF/IKK binding domain but not the kinase domain. NIK-aly does not interact with IKKα, and previous studies have suggested that this residue is also required for inhibition of LMP1-mediated NF-κB by DNNIK. NIK(K-A) expresses the full-length NIK with two mutations at Lys429 and Lys430 in the kinase domain (K429A/K430A), which abrogates its kinase activity (31, 51). Using an antibody to the carboxy terminus of NIK, expression of all three NIK mutants was detected with high levels of the kinase-dead NIK(K-A). Transfection of NIK(K-A), but not DNNIK or

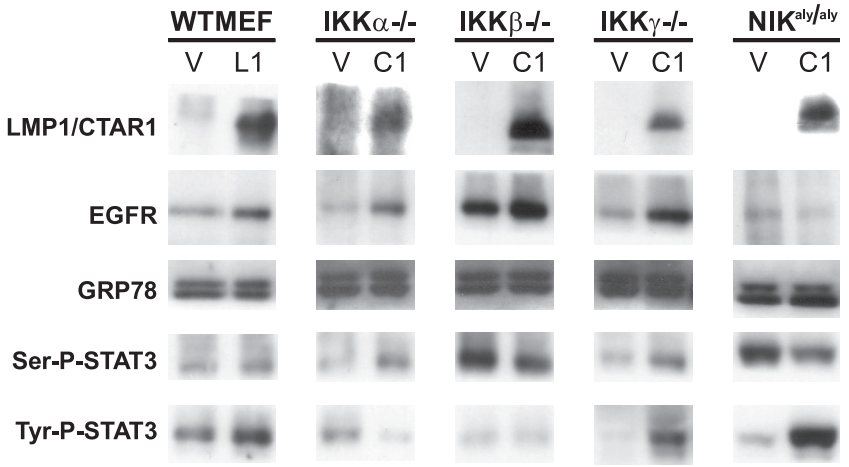

FIG. 2. LMP1-mediated EGFR upregulation is NIK dependent. Full-length LMP1 or CTAR1 was transduced or transfected into wild-type MEF cells or MEF cells defective for IKKα, IKKβ, IKKγ, or NIK. Expression of LMP1 or CTAR1 was confirmed by immunoblotting with specific antibody against HA tag (wild-type, IKKβ$^{-/-}$, and NIK$^{aly/aly}$ MEF cells) or Myc tag (IKKα$^{-/-}$ and IKKγ$^{-/-}$ MEF cells). Expression levels of EGFR, serine-phosphorylated STAT3, or tyrosine-phosphorylated STAT3 were analyzed by immunoblotting. Expression of GRP78 was measured by immunoblotting as a loading control. V, vector control; L1, LMP1; C1, CTAR1. The data are representative of at least three independent experiments.

NIK-aly, reduced CTAR1-induced EGFR expression significantly, by 35%. NIK(K-A) also decreased CTAR1-induced serine phosphorylation of STAT3 by 34%, while DNNIK and NIK-aly did not have significant effects. These findings indicate that NIK contributes to LMP1-mediated STAT3 serine phosphorylation and EGFR induction but not through its interaction with IKKα.

To determine whether NIK itself is sufficient to induce Ser-pSTAT3 and EGFR upregulation, wild-type NIK was transiently transfected into both C33A and 293T cells and EGFR expression and Ser-pSTAT3 were assessed by Western blot analysis (Fig. 3B). NIK expression was clearly detected in C33A and 293T cells. However, overexpression of NIK did not induce Ser-pSTAT3 or EGFR expression. In C33A cells, transfection of 4 μg of NIK very slightly increased Ser-pSTAT3, but it failed to induce expression of EGFR.

It is possible that activation of additional pathways by CTAR1 in combination with NIK is required to induce STAT3 activation and EGFR expression. Therefore, increasing amounts of the WT NIK construct were transfected into CTAR1-expressing NIK$^{aly/aly}$ MEF cells. Expression of CTAR1 was confirmed by Western blot analysis using antibody to the HA tag, and the effects on EGFR and Ser-pSTAT3 were determined (Fig. 3C). Similarly to the results observed in 293T and C33A cells, overexpression of NIK in NIK$^{aly/aly}$ MEF cells was not sufficient to induce either Ser-pSTAT3 or EGFR expression, even in the presence of CTAR1. These results suggest that although NIK is required for CTAR1-mediated EGFR upregulation, NIK overexpression alone is not sufficient to induce these pathways, and additional factors or processes are required for Ser-pSTAT3 and EGFR induction.

CTAR1-mediated p50 activation is not dependent on the canonical or noncanonical NF-κB pathway. LMP1 induction of EGFR transcription is mediated through effects on Bcl-3 and induction of p50 homodimers (24, 52, 53). To determine the requirement for specific IKK complexes on CTAR1-mediated p50 activation, CTAR1-expressing wild-type, IKKβ^{-}, IKKγ^{-}, and NIK$^{aly/aly}$ MEF cells were fractionated, and the nuclear lysates were analyzed to detect nuclear expression of p65, p50, and RelB (Fig. 4). The expression of PARP was analyzed as a loading control for the nuclear lysates. Nuclear translocation of p65 is characteristic of the canonical pathway, and p65 was detected in the nuclear lysates from WT, IKKγ^{-}, and NIK$^{aly/aly}$ MEFs but not in those from IKKβ-defective cells. Nuclear RelB is the major form of NF-κB that represents the noncanonical pathway, and CTAR1 induced nuclear translocation of RelB in wild-type and IKKβ-defective MEF cells but not in NIK-defective cells. These findings confirm that CTAR1-mediated translocation of p65 reflects activation of the canonical pathway and that the effects of CTAR1 on RelB require the noncanonical pathway. In contrast, the nuclear expression of p50 was significantly induced by CTAR1 in IKKβ^{-}, IKKγ^{-}, and NIK$^{aly/aly}$ MEF cells. This indicates that CTAR1-mediated p50 activation and nuclear translocation are distinct from its effects on either the canonical and noncanonical NF-κB pathways. A high basal level of nuclear p50 was detected in wild-type MEF cells and may be linked to the higher level of EGFR in WT MEFs and the slight effects of LMP1 on EGFR expression compared to that in C33A cells.

LMP1-mediated p50 activation is proteasome dependent. The mechanisms responsible for p50 activation have not been clearly defined. Several studies have indicated that p50 can be activated by a proteasome-dependent mechanism that either modulates p105 processing or impairs ribosomal progression (22). To determine whether p50 is activated by CTAR1 through proteasome-mediated mechanisms, C33A cells stably transduced with vector or CTAR1 were treated with dimethyl sulfoxide (DMSO) or a proteasome inhibitor, MG132 (26S inhibitor) or *clasto*-lactacystin β-lactone (20S inhibitor) (Fig. 5). The effects of these inhibitors on p105/50 were assessed by immunoblotting using anti-p105/50 antibody. β-Catenin is a known target of proteasomal degradation and was analyzed as a positive control for inhibition of proteasome activity.

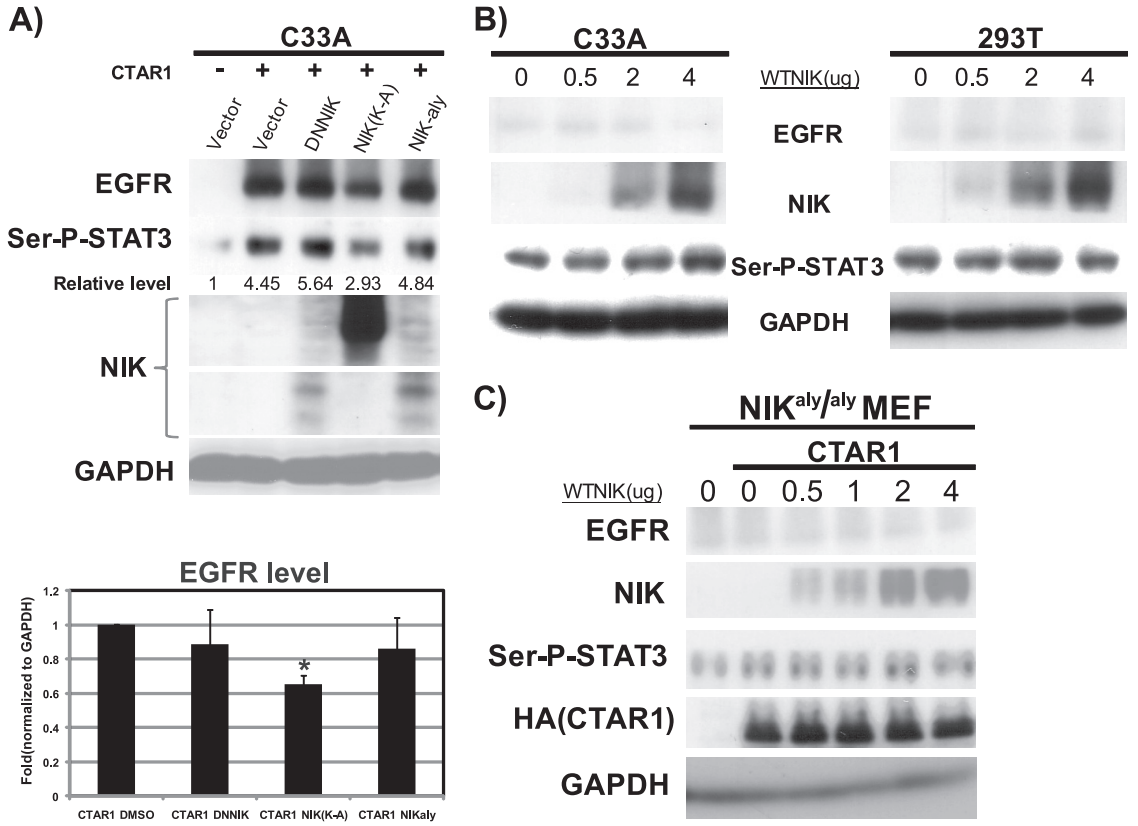

FIG. 3. Blocking NIK reduces LMP1-induced EGFR expression, but overexpression of NIK is not sufficient to mimic LMP1-mediated EGFR upregulation. (A) C33A cells stably expressing CTAR1 were transfected with pCDNA3 control vector or a dominant negative NIK construct (DNNIK), the DNNIK construct with an aly mutation (G860R; NIK-aly), or kinase-dead NIK [NIK(K-A)]. Expression levels of serine-phosphorylated STAT3 and EGFR were analyzed by immunoblotting. Expression of the NIK mutants was detected by immunoblotting using antibody against the C terminus of NIK. GAPDH was measured by immunoblotting as a loading control. The intensities of Ser-P-STAT3 and EGFR were quantitated using Image J software and normalized to the expression level of GAPDH from three independent experiments. *, $P < 0.05$. (B) C33A and 293T cells were transfected with increasing amounts of the wild-type NIK expression construct. (C) $NIK^{aly/aly}$ MEF cells stably expressing CTAR1 were transfected with increasing amounts of the wild-type NIK expression construct. CTAR1 expression was confirmed by immunoblotting with antibody against the HA tag. The expression of NIK, EGFR, and serine-phosphorylated STAT3 was analyzed by immunoblotting. Expression of GAPDH was measured by immunoblotting as a loading control. The images shown in panels B and C are representative of three independent experiments.

GAPDH and emerin were analyzed as loading controls for whole-cell lysates and nuclear lysates, respectively. In CTAR1-expressing C33A cells where nuclear expression of p50 was significantly induced, treatment with 1 μM or 10 μM MG132 reduced the p50 translocation level by 72% and 64%, respectively (Fig. 5A). Similarly, treatment with 1 μM or 10 μM *clasto*-lactacystin β-lactone reduced CTAR1-induced p50 nuclear translocation by 35% and 69%, respectively. Nuclear p50 was not detected in the C33A cells transduced with the pBabe control plasmid, and treatment with proteasome inhibitors did not have a significant effect (data not shown). This result indicates that LMP1-CTAR1 induction of p50 nuclear translocation is mediated through effects on proteasome activity. β-Catenin expression was slightly higher in C33A cells expressing LMP1, which is consistent with the described effects of LMP1 on junctional proteins (48). In cells treated with proteasome inhibitors, β-catenin levels were increased in control and CTAR1-expressing cells and higher-molecular-weight forms of β-catenin were detected, reflecting its ubiquitination and the successful inhibition of proteasomal activity (Fig. 5B).

The level of p105-to-p50 processing was determined by calculating the ratio of p50 to the total p105/50 complex in whole-cell lysates of C33A cells transduced with pBabe control or CTAR1 (Fig. 5B). The processing of p105 to p50 was enhanced by approximately 15% in CTAR1-expressing C33A cells compared to pBabe control cells. After MG132 treatment, the percentage of processed p50 was not significantly affected in either vector control or CTAR1-expressing cells. Treatment with the 20S proteasomal inhibitor *clasto*-lactacystin β-lactone did not affect p50 processing in pBabe control cells. However, treatment with 10 μM *clasto*-lactacystin β-lactone reduced p50 processing in CTAR1-expressing cells to approximately 40%, which is similar to the level observed in pBabe control cells. These findings indicate that proteasome-dependent mechanisms contribute in part to LMP1 effects on p50 processing. The effects of proteasome inhibition on the levels of nuclear p50 are much more evident than those on p105 processing in

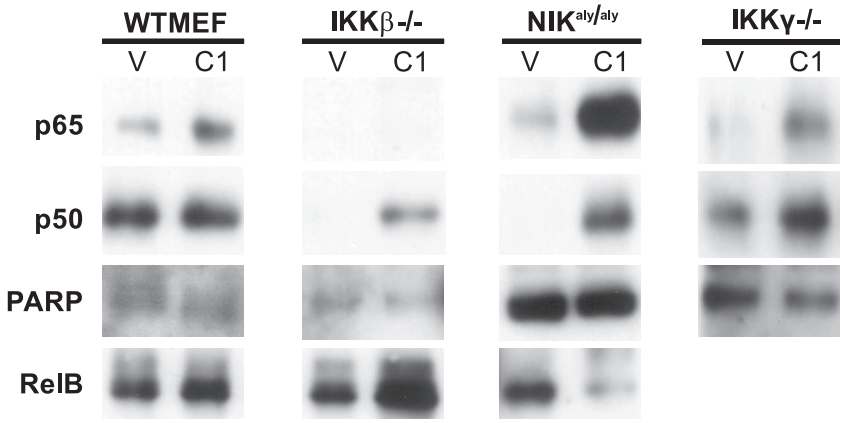

FIG. 4. CTAR1-mediated p50 activation is not dependent on the canonical or noncanonical NF-κB pathway. Wild-type, IKKβ$^{-/-}$, IKKγ$^{-/-}$, and NIK$^{aly/aly}$ MEF cells stably transduced with vector or CTAR1 were fractionated, and nuclear lysates were subjected to immunoblot analysis with antibodies specifically against p65 (RelA), p50, and RelB. PARP expression was analyzed by immunoblotting as a loading control. Data are representative of at least two independent experiments. V, vector control; C1, CTAR1.

whole-cell lysates. This suggests that the small increased amount of p50 is effectively transported to the nucleus.

LMP1-mediated EGFR induction requires TRAF2 and TRAF3 but not TRAF6. Initial studies showed that the TRAF binding motif of LMP1 was required for CTAR1-induced EGFR expression and NF-κB activation, and these effects were decreased by dominant negative TRAF2 and TRAF3 (35, 37). To further evaluate this requirement, LMP1 and LMP1-CTAR1 were expressed in MEF cells deficient for TRAF2, TRAF3, or TRAF6 (Fig. 6). The expression levels of EGFR, LMP1, and deletion mutants were determined using immunoblotting of whole-cell lysates (Fig. 6A). LMP1 and CTAR1 were detected using antibodies against the HA or Myc epitope tags. Interestingly, the absence of specific TRAFs apparently affects LMP1 modification such that the predominant LMP1 bands differ in size. EGFR was expressed at high levels in TRAF2$^{-/-}$ MEFs, intermediate levels were expressed in TRAF3$^{-/-}$ MEFs, and it was barely detected in TRAF6$^{-/-}$ MEFs. This confirmed that the presence and absence of TRAFs modulate expression of the EGFR. Expression of LMP1 or CTAR1 changed these patterns of expression. In the TRAF2-null and TRAF3-null MEFs, LMP1 or CTAR1 expression did not increase EGFR expression. In contrast, in the TRAF6-null MEFs, LMP1 and CTAR1 clearly induced EGFR expression (Fig. 6A). The ability of LMP1-CTAR1 to induce EGFR in TRAF6-null MEFs but not in TRAF2- or TRAF3-null MEFS is consistent with previously identified interactions of CTAR1 with TRAF2 and TRAF3 but not with TRAF6.

By immunoblot analysis, the nuclear level of p50 was analyzed in TRAF2$^{-/-}$, TRAF3$^{-/-}$, and TRAF6$^{-/-}$ MEFs, and the serine phosphorylation of STAT3 was analyzed in TRAF2$^{-/-}$ and TRAF3$^{-/-}$ MEF cells (Fig. 6B). Compared to the vector control cells, CTAR1 induced nuclear expression of p50 in TRAF3$^{-/-}$ and TRAF6$^{-/-}$ cells but did not affect nuclear p50 levels in TRAF2$^{-/-}$ MEF cells that had elevated basal nuclear p50. In contrast, CTAR1 was able to increase STAT3 serine phosphorylation in TRAF2$^{-/-}$ but not TRAF3$^{-/-}$ MEF cells that had an elevated basal level of STAT3 serine phosphorylation. These results indicate that TRAF2 and TRAF3 contribute to CTAR1-mediated EGFR induction through possible effects on p50 activation and STAT3 serine phosphorylation, respectively.

DISCUSSION

LMP1 is a member of the TNFR family and potently activates NF-κB transcriptional activity. Early studies identified two distinct NF-κB-activating domains in the carboxy terminus, CTAR1 and CTAR2, and determined that CTAR2 had greater activity in NF-κB reporter assays (18, 42). However, identification of the specific complexes using electrophoretic mobility shifts assay (EMSA) revealed that CTAR1 activated multiple forms of NF-κB while CTAR2 primarily activated a complex containing p65 (35). In addition, it was shown that CTAR1 significantly enhanced processing of p100 to p52 (42). Subsequent studies have further characterized activation of NF-κB and defined the canonical pathway, which involves the IKK complex containing IKKα, IKKβ, and IKKγ, to phosphorylate and negatively regulate the inhibitor of NF-κB, IκBα. It is now known that CTAR2 activates the IKKγ (NEMO)/IKKβ-dependent canonical NF-κB pathway through TRAF6/IRAK1/TAK1 (29, 46, 57). CTAR1, on the other hand, activates the IKKγ (NEMO)-independent but NIK/IKKα-dependent noncanonical NF-κB pathway, in addition to causing some activation of the canonical pathway (1, 9, 30). An atypical IKKβ-dependent/IKKγ-independent NF-κB activation has also been previously described in LMP1-expressing cell culture models (30). Our previous studies have identified an additional LMP1-mediated activation of NF-κB, in which CTAR1 of LMP1 activates two NF-κB members, p50 and Bcl-3, that are induced to bind to the EGFR promoter by LMP1 and increase EGFR transcription (24, 52, 53). The data in this study indicate that this novel pathway is independent of factors required for activation of the canonical or noncanonical NF-κB pathways. IKKα, IKKβ, and IKKγ, all of which are important components in the canonical NF-κB pathway, were not required for CTAR1-induced EGFR expression (Fig. 2). This result is consistent with previous data indicating that CTAR2 alone was not

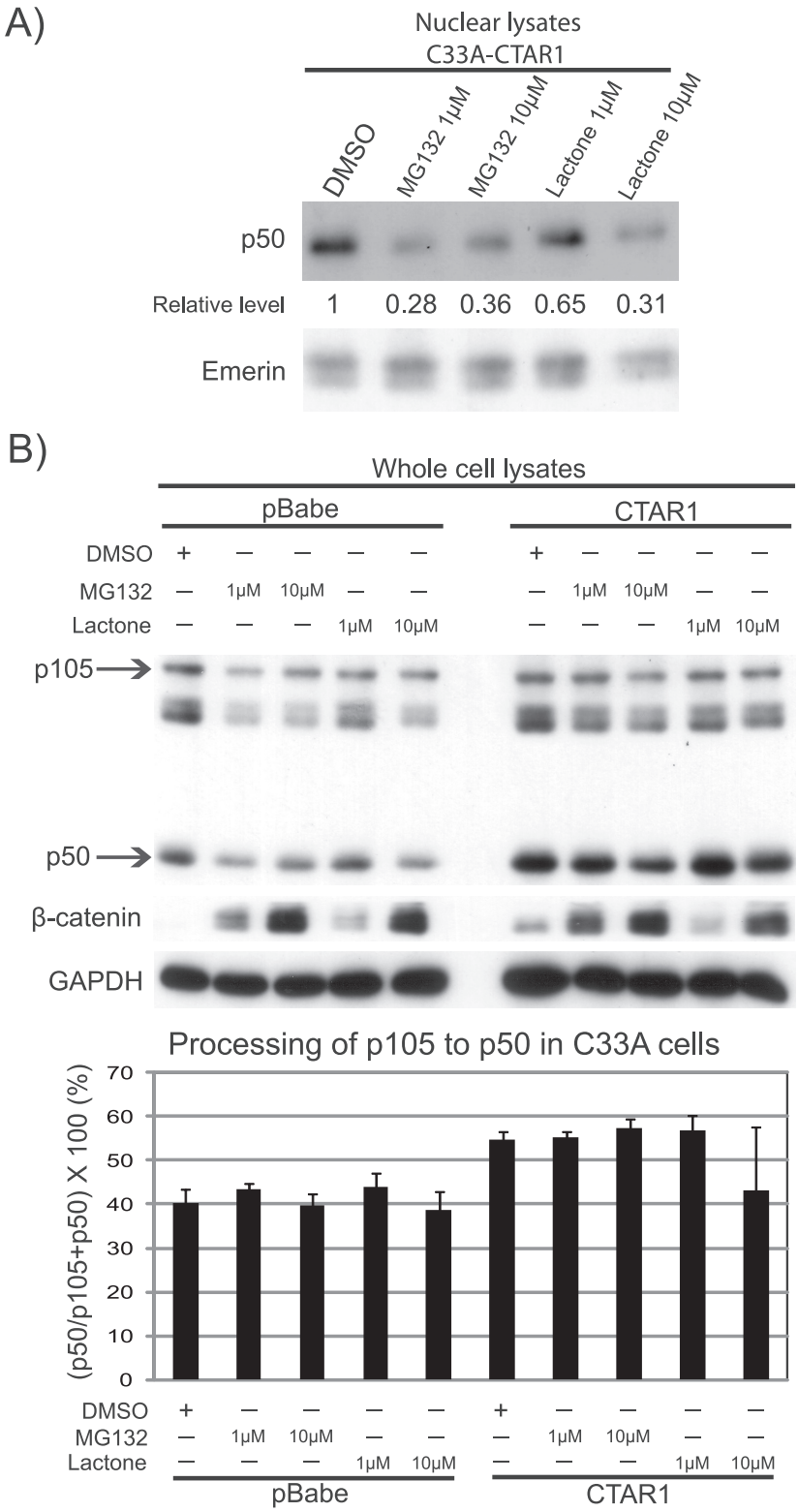

FIG. 5. CTAR1 mediates p50 activation through proteasome-dependent pathways. C33A cells stably transduced with vector control or CTAR1 were treated with DMSO or the proteasome inhibitor MG132 or β-lactone (1 or 10 μM) for 5 h and subjected to fractionation. Nuclear lysates (A) and whole-cell lysates (B) of these cells were analyzed by immunoblotting. Antibody against p105/50 was used to detect the processing and activation of p50. Expression of GAPDH and emerin was detected as loading controls for whole-cell lysates and nuclear lysates, respectively. The intensities of p105/50 bands were quantitated using Image J software, and the percentage of processed p50 in whole-cell lysates was calculated as [p50/(p50 + p105)] × 100%. The level of p50 nuclear expression was quantitated by normalizing p50 to the expression level of emerin. The data are the averages and standard errors from three independent experiments.

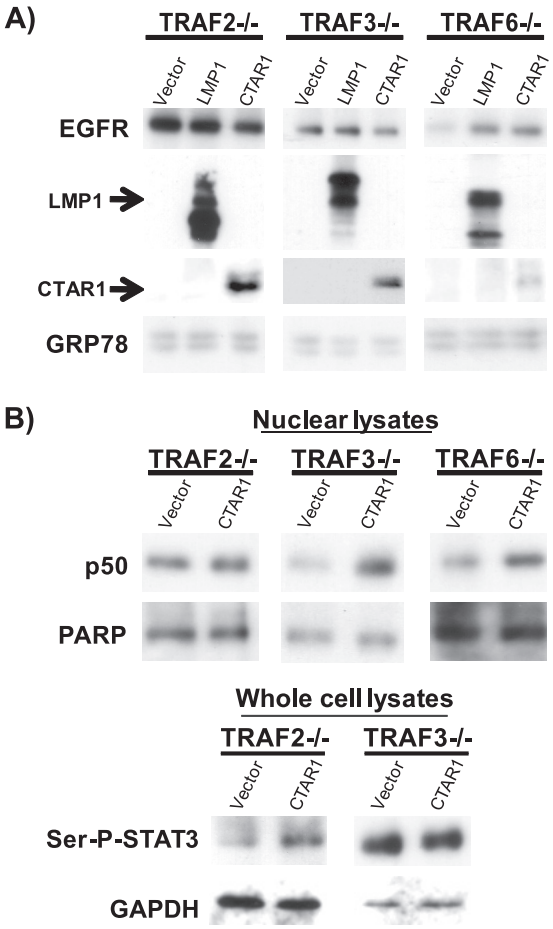

FIG. 6. LMP1-mediated EGFR upregulation is TRAF2 and TRAF3 dependent but not TRAF6 dependent. (A) TRAF2$^{-/-}$, TRAF3$^{-/-}$, and TRAF6$^{-/-}$ MEF cells were transfected (TRAF3$^{-/-}$) or transduced with vector control, full-length LMP1, or CTAR1, and whole-cell lysates of these cells were subject to immunoblotting with EGFR-specific antibodies. Expression of LMP1 or CTAR1 was confirmed by immunoblotting with specific antibody against full-length LMP1 (S12), HA tag, or Myc tag (TRAF3$^{-/-}$). Expression of GRP78 was detected as loading control. (B) TRAF2$^{-/-}$, TRAF3$^{-/-}$, and TRAF6$^{-/-}$ MEF cells expressing CTAR1 were fractionated. Nuclear and whole-cell lysates were analyzed by immunoblotting with p50 (TRAF2$^{-/-}$, TRAF3$^{-/-}$, and TRAF6$^{-/-}$ cells) and Ser-P-STAT3 (TRAF2$^{-/-}$ and TRAF3$^{-/-}$ cells) antibodies, respectively. Expression of PARP and GAPDH was detected as loading controls. The results shown are representative of at least two independent experiments.

able to induce EGFR expression despite its ability to activate strong canonical NF-κB signaling (Fig. 1) (24, 35).

Although the CTAR1-mediated EGFR induction was inhibited in NIK$^{aly/aly}$ cells, perhaps suggesting a requirement for the noncanonical NF-κB pathway, CTAR1-mediated induction of EGFR did not require IKKα, the functional kinase in the noncanonical NF-κB pathway. The decreased EGFR induction by the dominant negative NIK construct, kinase-dead NIK(K-A), in CTAR1-expressing C33A cells suggests that NIK does contribute to CTAR1-mediated EGFR induction (Fig. 3A). In contrast to NIK(K-A), the truncated DNNIK and NIK-aly did not significantly reduce CTAR1-induce STAT3 serine phosphorylation or EGFR expression, although both constructs still contain the TRAF/IKK binding domain. These findings indicate that the N terminus of NIK is an important contributing factor in LMP1-mediated EGFR induction. NIK-aly has previously been shown not to inhibit LMP1 activation of an NF-κB reporter, and LMP1 also activated the NF-κB reporter in the NIK$^{aly/aly}$ MEFs. The lack of an effect of NIK-aly on CTAR1-induced EGFR expression suggests that specific protein interactions and functions mediated through the aly residue (G860R), other than IKKα interaction, are important for EGFR upregulation. This result is consistent with the observation that CTAR1 could induce EGFR expression in IKKα-defective MEFs but not in NIK$^{aly/aly}$ MEF cells. Despite the requirement for NIK in LMP1-mediated EGFR induction, overexpression of NIK was not sufficient to mimic CTAR1-mediated STAT3 activation and EGFR upregulation in epithelial cells, and overexpression of wild-type NIK in the presence of CTAR1 did not induce Ser-pSTAT3 or EGFR induction in NIK-defective cells (Fig. 3B and C). These findings suggest that additional NIK-associated factors or NIK-mediated effects on other kinases contribute to this pathway and that a balance of expression level between NIK and NIK-associated factors may be required. These findings are similar to the requirement for TRAF3 for LMP1 signaling. Although TRAF3 is required for LMP1-mediated signaling pathways and transformation, overexpression of TRAF3 in LMP1 expression cells impairs CTAR1-mediated NF-κB activation (2, 33, 35, 37). TRAF3 binds strongly to CTAR1, and its overexpression likely alters the components in the LMP1/TRAF complexes that activate NF-κB.

It is known that both TRAF3 and TRAF2 regulate NIK, and it is thought that TRAF3 is required to form a complex containing TRAF2 and cIAP that induces NIK turnover (54). This suggests a negative regulatory mechanism between TRAF2/TRAF3 and NIK. However, CTAR1 failed to induce EGFR expression in MEF cells defective for TRAF2, TRAF3, or NIK. It is possible that LMP1-CTAR1 may mediate EGFR upregulation through NIK by sequestering the NIK regulators TRAF2 and TRAF3. This hypothesis would be consistent with the ability of LMP1 to modulate formation of TRAF-containing complexes and their effects on various targets.

The other important member of the EGFR-inducing complex, p50, is activated and translocated into the nucleus in LMP1/CTAR1-expressing C33A cells (24, 42, 53). Several mechanisms have been suggested to mediate p50 activation. This study reveals that CTAR1-induced p50 activation is not dependent on either the canonical or noncanonical NF-κB pathway, since this occurred in IKKβ-, IKKγ-, and NIK-defective MEF cells (Fig. 4). Interestingly, CTAR1 did not induce significant p50 activation in WT MEF cells. This may be due to the relatively high level of nuclear p50 in WT MEF cells and may partially explain why LMP1 only slightly induced EGFR expression in WT MEF cells compared to C33A or other mutant MEF cells.

Multiple studies have suggested that p50 can be activated through processing of its precursor, p105. This processing could occur in ubiquitination-dependent or ubiquitination-independent fashion (3, 5, 22, 38). It was also suggested that p50 could be generated through a mechanism involved cotranslational ribosome halting (27, 28). One consensus among these

suggested mechanisms is that they are all proteasome dependent. The data presented here indicate that CTAR1 does enhance processing of p50, which is consistent with previous studies (42). Proteasome inhibition with either 26S or 20S inhibitor reduced nuclear p50 in CTAR1-expressing cells (Fig. 5). Interestingly, both the 26S inhibitor MG132 and the 20S inhibitor *clasto*-lactacystin β-lactone inhibited CTAR1-induced nuclear translocation of p50, with only a slight effect on processing detected by inhibition of the 20S proteasome. This suggests that the slightly increased levels of processed p50 are efficiently transported to the nucleus (38). It is likely that LMP1 regulates multiple pathways to mediate p50 activation. This is supported by the observation that CTAR1 induced p50 activation in MEF cells defective for IKKβ, which has been suggested as the kinase responsible for p105 processing (5). Several proteins have been suggested previously to regulate p50 translocation, including Bcl-3 and importin-3α (11, 59). The fact that LMP1-CTAR1 induces Bcl-3 expression and nuclear localization makes it an attractive candidate (24, 40). It will be of interest to determine if Bcl-3 and importins contribute to CTAR1-mediated p50 activation and induction of EGFR.

LMP1-associated TRAFs are important for LMP1-mediated signaling pathways, including JNK, p38, and NF-κB activation (50). Our initial studies indicated that the TRAF binding motif of CTAR1 was required for LMP1 induction of EGFR expression (35). Expression of CTAR1 in MEF cells defective for different TRAFs revealed that TRAF2 and TRAF3, but not TRAF6, are required for CTAR1-mediated EGFR induction (Fig. 6). This result is consistent with the findings that TRAF2 and TRAF3, but not TRAF6, interact with the CTAR1 TRAF binding motif. Although TRAF6 does not interact with CTAR1, it has been shown that TRAF6 can interact with NIK (6). It is possible that the interaction of CTAR2 with TRAF6 modulates the TRAF6 interaction with NIK and NIK-regulated activation of Ser-pSTAT3 or downstream kinases. LMP1 containing CTAR1 but deleted for CTAR2 usually has elevated effects in comparison to full-length LMP1 in C33A cells (24). The effects of the two domains on various TRAF-containing complexes are likely the basis for the enhanced activity of CTAR1 in the absence of CTAR2. It is also interesting to note that another potential STAT3 kinase, extracellular signal-regulated kinase (ERK), has been shown to be regulated by NIK and that CTAR1-mediated ERK activation and transformation were also inhibited by dominant negative TRAF2 or TRAF3, but not TRAF6 (12, 33). The interaction of LMP1 with TRAF2 and TRAF3 likely activates multiple potentially redundant kinases that affect transcription factors that regulate EGFR expression.

In conclusion, this study reveals that LMP1-CTAR1 induces EGFR expression through NIK, TRAF2, and TRAF3 independently of traditionally defined canonical and noncanonical NF-κB pathways. Two important EGFR-inducing factors, STAT3 and p50, are regulated distinctively in the presence or absence of various IKK and TRAF molecules. The effects of LMP1 on the Ser-pSTAT3 potentially require NIK and TRAF3, whereas the effects on p50 require TRAF2 but are independent of NIK, TRAF3, and TRAF6. The data suggest that distinct combinations of LMP1-activated effectors regulate the effects of LMP1 expression and its ability to induce transformation. The further study of LMP1 and its activation of NF-κB and additional pathways will likely clarify the link between specific signal transducing complexes and the downstream effectors, as well as its contribution to EBV-associated cancer development.

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REFERENCES

1. **Atkinson, P. G., H. J. Coope, M. Rowe, and S. C. Ley.** 2003. Latent membrane protein 1 of Epstein-Barr virus stimulates processing of NF-kappa B2 p100 to p52. J. Biol. Chem. **278:**51134–51142.
2. **Bishop, G. A., and P. Xie.** 2007. Multiple roles of TRAF3 signaling in lymphocyte function. Immunol. Res. **39:**22–32.
3. **Ciechanover, A., H. Gonen, B. Bercovich, S. Cohen, I. Fajerman, A. Israel, F. Mercurio, C. Kahana, A. L. Schwartz, K. Iwai, and A. Orian.** 2001. Mechanisms of ubiquitin-mediated, limited processing of the NF-kappaB1 precursor protein p105. Biochimie **83:**341–349.
4. **Cogswell, P. C., D. C. Guttridge, W. K. Funkhouser, and A. S. Baldwin, Jr.** 2000. Selective activation of NF-kappa B subunits in human breast cancer: potential roles for NF-kappa B2/p52 and for Bcl-3. Oncogene **19:**1123–1131.
5. **Cohen, S., H. Achbert-Weiner, and A. Ciechanover.** 2004. Dual effects of IkappaB kinase beta-mediated phosphorylation on p105 Fate: SCF(beta-TrCP)-dependent degradation and SCF(beta-TrCP)-independent processing. Mol. Cell. Biol. **24:**475–486.
6. **Darnay, B. G., J. Ni, P. A. Moore, and B. B. Aggarwal.** 1999. Activation of NF-kappaB by RANK requires tumor necrosis factor receptor-associated factor (TRAF) 6 and NF-kappaB-inducing kinase. Identification of a novel TRAF6 interaction motif. J. Biol. Chem. **274:**7724–7731.
7. **Delhase, M., M. Hayakawa, Y. Chen, and M. Karin.** 1999. Positive and negative regulation of IkappaB kinase activity through IKKbeta subunit phosphorylation. Science **284:**309–313.
8. **Eliopoulos, A. G., S. M. Blake, J. E. Floettmann, M. Rowe, and L. S. Young.** 1999. Epstein-Barr virus-encoded latent membrane protein 1 activates the JNK pathway through its extreme C terminus via a mechanism involving TRADD and TRAF2. J. Virol. **73:**1023–1035.
9. **Eliopoulos, A. G., J. H. Caamano, J. Flavell, G. M. Reynolds, P. G. Murray, J. L. Poyet, and L. S. Young.** 2003. Epstein-Barr virus-encoded latent infection membrane protein 1 regulates the processing of p100 NF-kappaB2 to p52 via an IKKgamma/NEMO-independent signalling pathway. Oncogene **22:**7557–7569.
10. **Everly, D. N., Jr., B. A. Mainou, and N. Raab-Traub.** 2004. Induction of Id1 and Id3 by latent membrane protein 1 of Epstein-Barr virus and regulation of p27/Kip and cyclin-dependent kinase 2 in rodent fibroblast transformation. J. Virol. **78:**13470–13478.
11. **Fagerlund, R., L. Kinnunen, M. Kohler, I. Julkunen, and K. Melen.** 2005. NF-kappaB is transported into the nucleus by importin alpha3 and importin alpha4. J. Biol. Chem. **280:**15942–15951.
12. **Foehr, E. D., J. Bohuslav, L. F. Chen, C. DeNoronha, R. Geleziunas, X. Lin, A. O'Mahony, and W. C. Greene.** 2000. The NF-kappa B-inducing kinase induces PC12 cell differentiation and prevents apoptosis. J. Biol. Chem. **275:**34021–34024.
13. **Fujita, T., G. P. Nolan, H. C. Liou, M. L. Scott, and D. Baltimore.** 1993. The candidate proto-oncogene bcl-3 encodes a transcriptional coactivator that activates through NF-kappa B p50 homodimers. Genes Dev. **7:**1354–1363.
14. **Hacker, H., V. Redecke, B. Blagoev, I. Kratchmarova, L. C. Hsu, G. G. Wang, M. P. Kamps, E. Raz, H. Wagner, G. Hacker, M. Mann, and M. Karin.** 2006. Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. Nature **439:**204–207.
15. **Hayden, M. S., and S. Ghosh.** 2008. Shared principles in NF-kappaB signaling. Cell **132:**344–362.
16. **Hoberg, J. E., A. E. Popko, C. S. Ramsey, and M. W. Mayo.** 2006. IkappaB kinase alpha-mediated derepression of SMRT potentiates acetylation of RelA/p65 by p300. Mol. Cell. Biol. **26:**457–471.
17. **Huang, W. C., T. K. Ju, M. C. Hung, and C. C. Chen.** 2007. Phosphorylation of CBP by IKKalpha promotes cell growth by switching the binding preference of CBP from p53 to NF-kappaB. Mol. Cell **26:**75–87.
18. **Huen, D. S., S. A. Henderson, D. Croom-Carter, and M. Rowe.** 1995. The Epstein-Barr virus latent membrane protein-1 (LMP1) mediates activation of NF-kappa B and cell surface phenotype via two effector regions in its carboxy-terminal cytoplasmic domain. Oncogene **10:**549–560.

19. **Kaye, K. M., O. Devergne, J. N. Harada, K. M. Izumi, R. Yalamanchili, E. Kieff, and G. Mosialos.** 1996. Tumor necrosis factor receptor associated factor 2 is a mediator of NF-kappa B activation by latent infection membrane protein 1, the Epstein-Barr virus transforming protein. Proc. Natl. Acad. Sci. U. S. A. **93:**11085–11090.
20. **Kaye, K. M., K. M. Izumi, and E. Kieff.** 1993. Epstein-Barr virus latent membrane protein 1 is essential for B-lymphocyte growth transformation. Proc. Natl. Acad. Sci. U. S. A. **90:**9150–9154.
21. **Kieff, E., and A. B. Rickinson.** 2001. Epstein-Barr virus and its replication, p. 2511–2573. *In* D. M. Knipe (ed.), Field's virology, 4th ed., vol. 2. Lippincott Williams & Wilkins, Philadelphia, PA.
22. **Kravtsova-Ivantsiv, Y., S. Cohen, and A. Ciechanover.** 2009. Modification by single ubiquitin moieties rather than polyubiquitination is sufficient for proteasomal processing of the p105 NF-kappaB precursor. Mol. Cell **33:**496–504.
23. **Kulwichit, W., R. H. Edwards, E. M. Davenport, J. F. Baskar, V. Godfrey, and N. Raab-Traub.** 1998. Expression of the Epstein-Barr virus latent membrane protein 1 induces B cell lymphoma in transgenic mice. Proc. Natl. Acad. Sci. U. S. A. **95:**11963–11968.
24. **Kung, C. P., and N. Raab-Traub.** 2008. Epstein-Barr virus latent membrane protein 1 induces expression of the epidermal growth factor receptor through effects on Bcl-3 and STAT3. J. Virol. **82:**5486–5493.
25. **Laherty, C. D., H. M. Hu, A. W. Opipari, F. Wang, and V. M. Dixit.** 1992. The Epstein-Barr virus LMP1 gene product induces A20 zinc finger protein expression by activating nuclear factor kappa B. J. Biol. Chem. **267:**24157–24160.
26. **Li, Z. W., W. Chu, Y. Hu, M. Delhase, T. Deerinck, M. Ellisman, R. Johnson, and M. Karin.** 1999. The IKKbeta subunit of IkappaB kinase (IKK) is essential for nuclear factor kappaB activation and prevention of apoptosis. J. Exp. Med. **189:**1839–1845.
27. **Lin, L., G. N. DeMartino, and W. C. Greene.** 1998. Cotranslational biogenesis of NF-kappaB p50 by the 26S proteasome. Cell **92:**819–828.
28. **Lin, L., G. N. DeMartino, and W. C. Greene.** 2000. Cotranslational dimerization of the Rel homology domain of NF-kappaB1 generates p50-p105 heterodimers and is required for effective p50 production. EMBO J. **19:**4712–4722.
29. **Luftig, M., E. Prinarakis, T. Yasui, T. Tsichritzis, E. Cahir-McFarland, J. Inoue, H. Nakano, T. W. Mak, W. C. Yeh, X. Li, S. Akira, N. Suzuki, S. Suzuki, G. Mosialos, and E. Kieff.** 2003. Epstein-Barr virus latent membrane protein 1 activation of NF-kappaB through IRAK1 and TRAF6. Proc. Natl. Acad. Sci. U. S. A. **100:**15595–15600.
30. **Luftig, M., T. Yasui, V. Soni, M. S. Kang, N. Jacobson, E. Cahir-McFarland, B. Seed, and E. Kieff.** 2004. Epstein-Barr virus latent infection membrane protein 1 TRAF-binding site induces NIK/IKK alpha-dependent noncanonical NF-kappaB activation. Proc. Natl. Acad. Sci. U. S. A. **101:**141–146.
31. **Luftig, M. A., E. Cahir-McFarland, G. Mosialos, and E. Kieff.** 2001. Effects of the NIK aly mutation on NF-kappaB activation by the Epstein-Barr virus latent infection membrane protein, lymphotoxin beta receptor, and CD40. J. Biol. Chem. **276:**14602–14606.
32. **Mainou, B. A., D. N. Everly, Jr., and N. Raab-Traub.** 2005. Epstein-Barr virus latent membrane protein 1 CTAR1 mediates rodent and human fibroblast transformation through activation of PI3K. Oncogene **24:**6917–6924.
33. **Mainou, B. A., D. N. Everly, Jr., and N. Raab-Traub.** 2007. Unique signaling properties of CTAR1 in LMP1-mediated transformation. J. Virol. **81:**9680–9692.
34. **Mayo, M. W., and A. S. Baldwin.** 2000. The transcription factor NF-kappaB: control of oncogenesis and cancer therapy resistance. Biochim. Biophys. Acta **1470:**M55–M62.
35. **Miller, W. E., J. L. Cheshire, and N. Raab-Traub.** 1998. Interaction of tumor necrosis factor receptor-associated factor signaling proteins with the latent membrane protein 1 PXQXT motif is essential for induction of epidermal growth factor receptor expression. Mol. Cell. Biol. **18:**2835–2844.
36. **Miller, W. E., H. S. Earp, and N. Raab-Traub.** 1995. The Epstein-Barr virus latent membrane protein 1 induces expression of the epidermal growth factor receptor. J. Virol. **69:**4390–4398.
37. **Miller, W. E., G. Mosialos, E. Kieff, and N. Raab-Traub.** 1997. Epstein-Barr virus LMP1 induction of the epidermal growth factor receptor is mediated through a TRAF signaling pathway distinct from NF-kappaB activation. J. Virol. **71:**586–594.
38. **Moorthy, A. K., O. V. Savinova, J. Q. Ho, V. Y. Wang, D. Vu, and G. Ghosh.** 2006. The 20S proteasome processes NF-kappaB1 p105 into p50 in a translation-independent manner. EMBO J. **25:**1945–1956.
39. **Nadiminty, N., J. Y. Chun, Y. Hu, S. Dutt, X. Lin, and A. C. Gao.** 2007. LIGHT, a member of the TNF superfamily, activates Stat3 mediated by NIK pathway. Biochem. Biophys. Res. Commun. **359:**379–384.
40. **Nakamura, H., C. Ishii, M. Suehiro, A. Iguchi, K. Kuroda, K. Shimizu, N. Shimizu, K. Imadome, M. Yajima, and S. Fujiwara.** 2008. The latent membrane protein 1 (LMP1) encoded by Epstein-Barr virus induces expression of the putative oncogene Bcl-3 through activation of the nuclear factor-kappaB. Virus Res. **131:**170–179.
41. **Oh, S. M., S. H. Lee, B. J. Lee, C. W. Pyo, N. K. Yoo, S. Y. Lee, J. Kim, and S. Y. Choi.** 2007. A distinct role of neutrophil lactoferrin in RelA/p65 phosphorylation on Ser536 by recruiting TNF receptor-associated factors to IkappaB kinase signaling complex. J. Immunol. **179:**5686–5692.
42. **Paine, E., R. I. Scheinman, A. S. Baldwin, Jr., and N. Raab-Traub.** 1995. Expression of LMP1 in epithelial cells leads to the activation of a select subset of NF-kappa B/Rel family proteins. J. Virol. **69:**4572–4576.
43. **Raab-Traub, N.** 2002. Epstein-Barr virus in the pathogenesis of NPC. Semin. Cancer Biol. **12:**431–441.
44. **Rayet, B., and C. Gelinas.** 1999. Aberrant rel/nfkb genes and activity in human cancer. Oncogene **18:**6938–6947.
45. **Rowe, M., M. Peng-Pilon, D. S. Huen, R. Hardy, D. Croom-Carter, E. Lundgren, and A. B. Rickinson.** 1994. Upregulation of bcl-2 by the Epstein-Barr virus latent membrane protein LMP1: a B-cell-specific response that is delayed relative to NF-kappa B activation and to induction of cell surface markers. J. Virol. **68:**5602–5612.
46. **Saito, N., G. Courtois, A. Chiba, N. Yamamoto, T. Nitta, N. Hironaka, M. Rowe, N. Yamamoto, and S. Yamaoka.** 2003. Two carboxyl-terminal activation regions of Epstein-Barr virus latent membrane protein 1 activate NF-kappaB through distinct signaling pathways in fibroblast cell lines. J. Biol. Chem. **278:**46565–46575.
47. **Schmidt-Supprian, M., W. Bloch, G. Courtois, K. Addicks, A. Israel, K. Rajewsky, and M. Pasparakis.** 2000. NEMO/IKK gamma-deficient mice model incontinentia pigmenti. Mol. Cell **5:**981–992.
48. **Shair, K. H., C. I. Schnegg, and N. Raab-Traub.** 2009. Epstein-Barr virus latent membrane protein-1 effects on junctional plakoglobin and induction of a cadherin switch. Cancer Res. **69:**5734–5742.
49. **Siegler, G., B. Meyer, C. Dawson, E. Brachtel, J. Lennerz, C. Koch, E. Kremmer, E. Niedobitek, R. Gonnella, B. Z. Pilch, L. S. Young, and G. Niedobitek.** 2004. Expression of tumor necrosis factor receptor-associated factor 1 in nasopharyngeal carcinoma: possible upregulation by Epstein-Barr virus latent membrane protein 1. Int. J. Cancer **112:**265–272.
50. **Soni, V., E. Cahir-McFarland, and E. Kieff.** 2007. LMP1 TRAFficking activates growth and survival pathways. Adv. Exp. Med. Biol. **597:**173–187.
51. **Sylla, B. S., S. C. Hung, D. M. Davidson, E. Hatzivassiliou, N. L. Malinin, D. Wallach, T. D. Gilmore, E. Kieff, and G. Mosialos.** 1998. Epstein-Barr virus-transforming protein latent infection membrane protein 1 activates transcription factor NF-kappaB through a pathway that includes the NF-kappaB-inducing kinase and the IkappaB kinases IKKalpha and IKKbeta. Proc. Natl. Acad. Sci. U. S. A. **95:**10106–10111.
52. **Thornburg, N. J., R. Pathmanathan, and N. Raab-Traub.** 2003. Activation of nuclear factor-kappaB p50 homodimer/Bcl-3 complexes in nasopharyngeal carcinoma. Cancer Res. **63:**8293–8301.
53. **Thornburg, N. J., and N. Raab-Traub.** 2007. Induction of epidermal growth factor receptor expression by Epstein-Barr virus latent membrane protein 1 C-terminal-activating region 1 is mediated by NF-kappaB p50 homodimer/Bcl-3 complexes. J. Virol. **81:**12954–12961.
54. **Vallabhapurapu, S., A. Matsuzawa, W. Zhang, P. H. Tseng, J. J. Keats, H. Wang, D. A. Vignali, P. L. Bergsagel, and M. Karin.** 2008. Nonredundant and complementary functions of TRAF2 and TRAF3 in a ubiquitination cascade that activates NIK-dependent alternative NF-kappaB signaling. Nat. Immunol. **9:**1364–1370.
55. **Wang, D., D. Liebowitz, and E. Kieff.** 1985. An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells. Cell **43:**831–840.
56. **Wilson, J. B., W. Weinberg, R. Johnson, S. Yuspa, and A. J. Levine.** 1990. Expression of the BNLF-1 oncogene of Epstein-Barr virus in the skin of transgenic mice induces hyperplasia and aberrant expression of keratin 6. Cell **61:**1315–1327.
57. **Wu, L., H. Nakano, and Z. Wu.** 2006. The C-terminal activating region 2 of the Epstein-Barr virus-encoded latent membrane protein 1 activates NF-kappaB through TRAF6 and TAK1. J. Biol. Chem. **281:**2162–2169.
58. **Yamamoto, Y., U. N. Verma, S. Prajapati, Y. T. Kwak, and R. B. Gaynor.** 2003. Histone H3 phosphorylation by IKK-alpha is critical for cytokine-induced gene expression. Nature **423:**655–659.
59. **Zhang, Q., J. A. Didonato, M. Karin, and T. W. McKeithan.** 1994. BCL3 encodes a nuclear protein which can alter the subcellular location of NF-kappa B proteins. Mol. Cell. Biol. **14:**3915–3926.